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The Editor comments

A MATTER OF SEMANTICS

Scientists, perhaps more than any other group, are aware of the necessity for accuracy in the choice of terminology or phraseology used in transmitting information. It is not surprising, then, that they are distressed when advertising directed to them goes beyond a reasonable degree of accepted puffery, to the point of being misleading or even inaccurate.

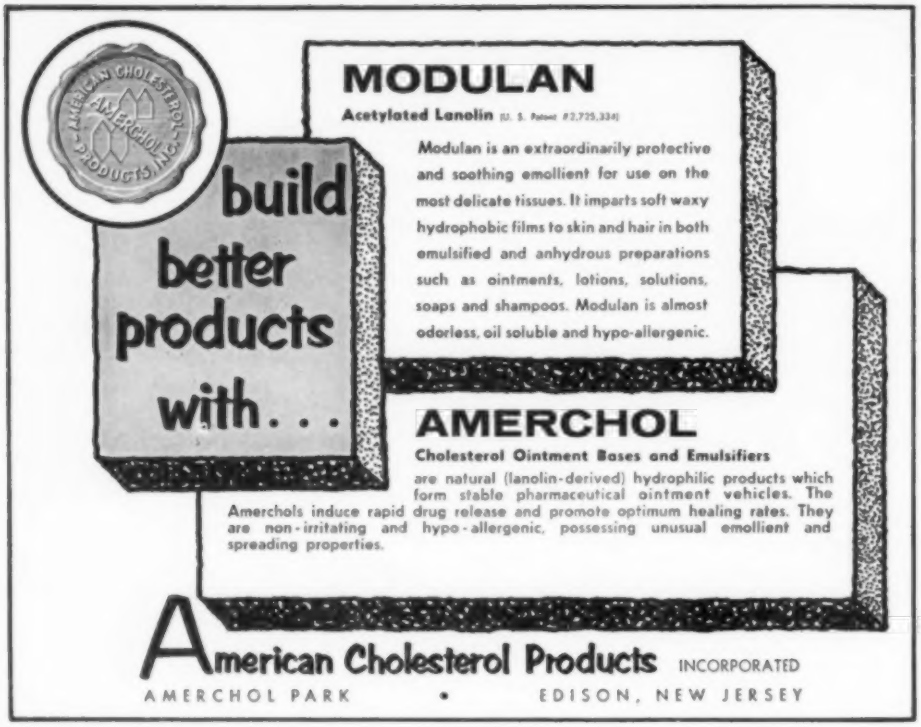
A case in point is a series of recent advertisements appearing in various chemical and pharmaceutical trade publications. These ads pertain to bulk chemicals and in very large, prominent type appears the statement **"FDA APPROVED."** In some cases, in much smaller type, this has been followed by ". . .for use as food additive." In other instances no such modifying phrase has appeared.

Under current food additive regulations, the Commissioner of Foods and Drugs may "authorize the use in foods of certain additives." In such cases, an order may be issued permitting, under specified conditions, the use of the particular item as a food additive. To describe such an item as *FDA approved* appears to be blatantly misleading, if not downright false. It suggests that the Food and Drug Administration has embarked upon a type of seal of acceptance program. It also implies a greater degree of safety for the chemical than may be justified on the basis of available pharmacological data.

It is our thought that such a designation is as objectionable as saying that a particular pharmaceutical is *FDA approved* because an effective New Drug Application has been issued for it; or in the case of a brand of aspirin, that it is *U. S. P. approved* simply because aspirin (acetylsalicylic acid) is listed in the *United States Pharmacopeia*. A similar comparison could be made with articles listed in the *National Formulary*.

Those who prepare advertising copy would do well to keep these points in mind when drafting future promotion for authorized food additives, and journals should review such wording in accepting these advertisements for publication.

Edward G. Feldmann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The next annual meeting of the American Pharmaceutical Association will be held in Las Vegas, Nevada, March 25–30, 1962. Titles and abstracts (not to exceed 200 words) of papers must be submitted to the appropriate Section secretary not later than January 1, 1962, or preferably earlier, in order to assure a place on any Section program. Papers should be sent to the appropriate Section secretary; the names and addresses of these secretaries are listed below.

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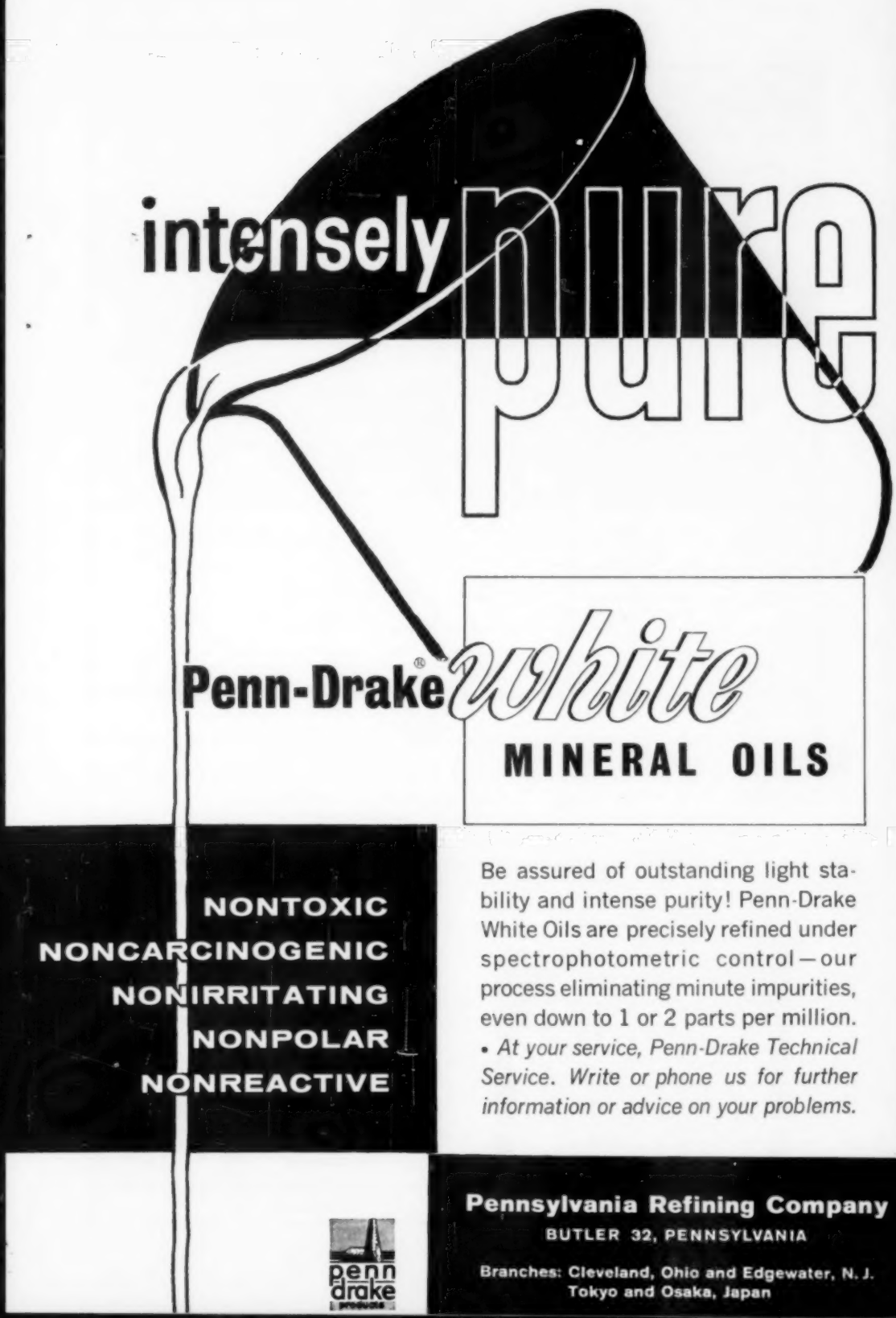
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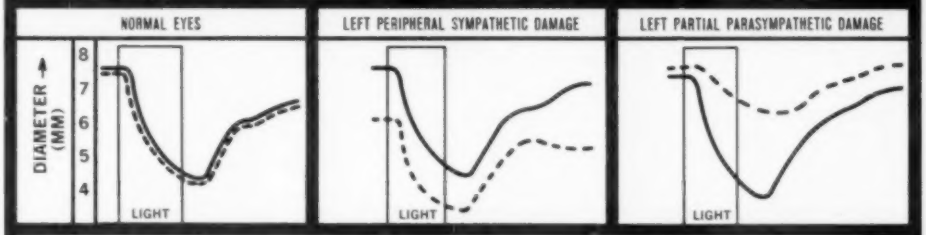

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JOURNAL OF **Pharmaceutical Sciences**

November 1961 volume 50, number 11

Review Article

Sulfurous Acid Salts as Pharmaceutical Antioxidants

By LOUIS C. SCHROETER

BISULFITES, pyrosulfites, and sulfites are employed as antioxidants in aqueous pharmaceutical preparations. The need for and the rational choice of antioxidants has been reviewed in detail in Schou's excellent review on oxidative degradation (1). Bisulfite was listed as a desirable antioxidant which was employed in many formulations. Typical pharmaceutical texts (2–4) refer to bisulfite as an antioxidant for compounds such as epinephrine, apomorphine, physostigmine, salicylates, etc.

Use of bisulfite, sulfite, and pyrosulfite salts as antioxidants in many formulations has led to many erroneous concepts regarding the relative efficacy of these salts. It is not uncommon to find references made to purported advantages of pyrosulfite over bisulfite in a liquid formulation and statements to the effect that sulfite is more reliable than bisulfite. Actually, the active antioxidant species in solution depends not on the form in which it is added, but on the final concentration of the thio compound and the final pH of the product. Sulfite antioxidants may be converted not only to sulfate but also to other species during the oxidative process. This often has a profound effect on the overall stability of the system and on the efficiency of the antioxidant. For these reasons, and because these compounds are not rigorously treated in usual inorganic textbooks, a brief discussion of sulfur oxy-acids has been included to help clarify the description of the oxidative mechanism.

Addition of bisulfites to formulations has given the desired antioxidant protection and clear, stable formulations have been prepared both in industry and hospitals. More important than a lack of knowledge of the mechanism of sulfite oxidation is the failure to appreciate the fact that the various sulfurous acid species can be reactants in many formulations. Thus, one may have a clear, pharmaceutically elegant formulation with little or no pharmacologic activity. Some of the typical bisulfite incompatibilities and representative "new" bisulfite reactions will be discussed.

CHEMISTRY

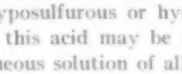
The relationship existing between sulfurous acid and its salts to other sulfur oxy-acids is presented on the basis of apparent oxidation states of the sulfur atom.

Sulfoxylic Acid.—This compound is not known in the pure state; however, a number of salts have been prepared, e.g., zinc dust and sulfonyl chloride react in ether to yield zinc sulfoxylate (5). The stable organic sulfones, R_2SO_2, are well known and sulfoxylic acid, H_2SO_2, may be considered as the simplest sulfone. The apparent oxidation state of sulfur in sulfox-

Received from the Pharmacy Research Section, Product Research and Development, The Upjohn Co., Kalamazoo, Mich.

The author wishes to thank Drs. J. G. Wagner, J. E. Tingstad, (The Upjohn Co.), B. J. Sciarrone (Rutgers University), T. D. W. Sokoloski (Washington State University), E. Nelson and S. Riegelman (University of California), H. B. Kostenbauder (Temple University), and E. G. Rippie (University of Minnesota) who offered suggestions during preparation of this manuscript.

ylic acid is +2. The electronic structure may be represented as

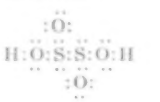


The redox characteristic (6) of the couple in acidic solution

$$H_2SO_2 + H_2O \rightleftharpoons H_2SO_3 + 2H^+ + 2e;\ E^0_{25} \cong -0.4\ v.$$

gives a measure of the ease of oxidation of sulfoxylic acid; this will serve as a useful basis for comparison with the other sulfur oxy-acids.

Dithionous Acid.—This compound is known variously as hyposulfurous or hydrosulfurous acid. Salts of this acid may be prepared by pouring an aqueous solution of alkali bisulfite through a Jones reductor (granulated zinc column). The apparent oxidation state of sulfur in dithionous acid, $H_2S_2O_4$, is +3; the electronic structure may be shown as



Spectral evidence appears to support a structure with the S—S bond, thus relating dithionite ion to dithionate and to pyrosulfite ion (7). Dithionites are stable as dry solids but solutions rapidly decompose, yielding bisulfite and thiosulfate

$$2S_2O_4^{2-} + H_2O \rightarrow S_2O_3^{2-} + 2HSO_3^-$$

Decomposition in acid yields sulfurous acid and sulfur as final products

$$H_2S_2O_4 \xrightarrow{[H^+]} H_2SO_3 + S + H_2O$$

Dithionites are powerful reducing agents (8) potentiometrically close to tin metal in acidic solution

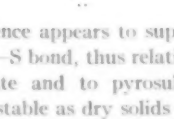
$$HS_2O_4^- + 2H_2O \rightleftharpoons 2H_2SO_3 + H^+ + 2e;\quad E^0_{25} = 0.23\ v.$$

$$S_2O_4^{2-} + 4OH^- \rightleftharpoons 2SO_3^{2-} + 2H_2O + 2e;\quad E^0_{25} = 1.4\ v.$$

Aqueous solutions of $Na_2S_2O_4$ are rapidly oxidized upon absorbing oxygen

$$S_2O_4^{2-} + O_2 + H_2O \rightleftharpoons HSO_4^- + HSO_3^-$$

Sulfurous Acid.—Solutions of sulfurous acid are readily obtained by introducing sulfur dioxide into water (9). The gas behaves as an acid in this reaction and the apparent oxidation state of the sulfur is +4. Sulfur dioxide is a nonlinear molecule which may be considered as tetra-functional in that it reacts not only as an acid as in the above reaction, but also as an oxidizing agent, base, and reducing agent. All of these functions have been observed (7, 8): SO_2 is about equal to divalent tin as a reductant (10); SO_2 acts as an oxidant with H_2S to yield elemental sulfur; SO_2 reacts as a base with boron trifluoride.



Dissolution of sulfur dioxide in water is a function of temperature and pressure as shown in Fig. 1 in which the weight of SO_2 per 100 Gm. of water is plotted as a function of temperature in the case when total pressure remains fixed at 1 atmosphere (11). The presence of unneutralized base, e.g., hydroxide ion, ephedrine, etc., will permit dissolution of more SO_2: the total amount will approximately equal that amount dissolved by the water plus the stoichiometric amount reacted with conjugate base in solution.

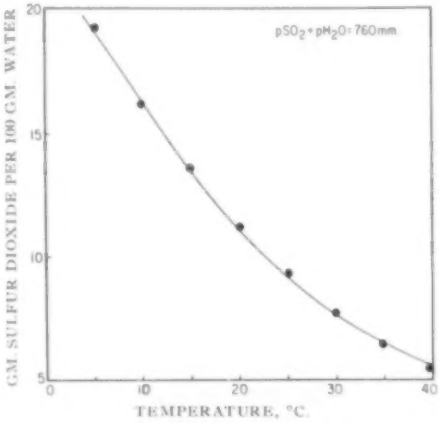
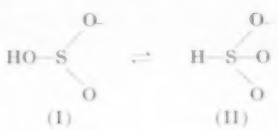


Fig. 1.—Grams of sulfur dioxide dissolved by 100 grams of water as a function of temperature when total pressure is maintained constant at 1 atmosphere.

Replacement of the first hydrogen of sulfurous acid by sodium ion yields sodium hydrogen sulfite or sodium bisulfite, $NaHSO_3$. The first ionization constant (thermodynamic) at 25°

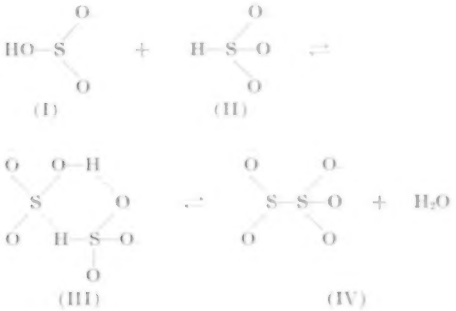
$$H_2SO_3 \rightleftharpoons H^+ + HSO_3^-;\ Ka_1\ 1.7 \times 10^{-2}$$

indicates that sulfurous acid is a relatively strong acid (12). Ultraviolet studies of bisulfite solutions (13) have shown the presence of four species in so-called bisulfite solutions (pH 3.5 to pH 5.5) depending on the concentration. Apparently the following equilibrium is involved at low concentration (3×10^{-3} *M*).



(I) (II)

The molar absorptivities of the isomeric bisulfite ions were determined: HSO_3^- (λ max. 215 mμ, log ε 1.78); $HOSO_2^-$ (λ max. 205.2 mμ, log ε apparent 2.75). At higher concentrations (10^{-2} *M*) the isomeric species tend to hydrogen bond to form a six-numbered ring structure (III) which, in turn, is in equilbrium with pyrosulfite (IV). Sciarrone (14) has pointed out the rather unusual nature of the proposed cyclic structure (III) in that the SH · · · · S hydrogen bond may be considered controversial and that the fortuitous ring gives little indication as to the mechanism of dehydration.



(I) (II) (III) (IV)

The log of the molar absorptivity of the pyrosulfite ion, $S_2O_5^{2-}$, was found to be 3.60 (λ max. 255 mμ), and the equilibrium constant for the conversion III ⇌ IV was evaluated $K = (S_2O_5^{2-})/(HSO_3^-)^2 = 7 \times 10^{-2}$ mole^{-1}.

Loss of the second hydrogen from sulfurous acid yields the doubly charged sulfite ion

$$HSO_3^- \rightleftharpoons H^+ + SO_3^{2-}; \quad Ka_2\ 6.24 \times 10^{-8}$$

The ionization constant above represents a thermodynamic value, i.e., results obtainced at infinite dilution at 25° (12). It must be remembered that the ionization tends to increase with increasing temperatures, e.g., autoclaving procedures or high temperature stability studies. The apparent ionization constant is also increased in typical formulations where ionic strength effects may be considerable. Determination of apparent ionization constants of sulfurous acid in solutions of high ionic strength or at high temperatures requires great care because of the pronounced tendency toward autoxidation by the sulfite species.

Redox characteristics (6, 15) of sulfurous acid are summarized by the couples

acid solution:

$$H_2SO_3 + H_2O \rightleftharpoons SO_4^{2-} + 4H^+ + 2e; \quad E^0_{25} = -0.2$$

basic solution:

$$H_2SO_3 + 4OH^- \rightleftharpoons SO_4^{2-} + 3H_2O + 2e; \quad E^0_{25} = +0.9$$

Solubilities of sulfite salts (16) of group II-A elements show a marked decrease from those of group I-A elements as shown in Table I.

Table I.—Solubility of Sulfite Salts of Group I-A and II-A Elements in Water at 25°

		Formula	SO_2, %	Solubility, Gm./100 ml.
I-A:	Na	$Na_2SO_3 \cdot 7H_2O$	25.41	24
	K	$K_2SO_3 \cdot 2H_2O$	32.97	100
II-A:	Ca	$CaSO_3 \cdot H_2O$	41.07	0.004
	Sr	$SrSO_3$	38.16	0.004
	Ba	$BaSO_3$	29.47	0.010

Sulfites are never added to pharmaceutical formulations in the form of strontium or barium salts and only rarely is the calcium salt employed. More often, the relatively insoluble calcium sulfite is formed *in situ* as the result of a metathetical reaction between alkali sulfite and a calcium salt of some anionic drug.

Analytical Methods.—Bisulfites, sulfites, and thiosulfates are commonly determined by iodometric titration (17). Thiosulfate may be directly titrated with iodine solution while bisulfites and sulfites are determined by adding excess iodine solution and back titrating with thiosulfate. The presence of small amounts of either sulfites or thiosulfates in solution may be quickly determined by various spot tests (18). The antimony chloride spot test is especially useful in that it permits the detection of thiosulfate in sulfite solutions (19). Potentiometric and polarographic methods may be utilized for quantitative determination of mixtures of the sulfur oxy-acid anions.

Bisulfites, sulfites, and pyrosulfites may be determined gravimetrically as the insoluble calcium sulfite (20); the method also serves for the determination of sulfites in the presence of thiosulfate (21). Acid-washed alumina has also been used to separate mixtures of these anions (22). Basic anion exchange resins offer possibilities for separation of various sulfur oxy-acid anions (23). Ultraviolet spectrophotometric studies have been used to determine bisulfite in the presence of pyrosulfite in aqueous solutions (13). The presence of bisulfite, etc., in powders or dry residues may be quantitatively determined by X-ray powder diffraction studies since most spectra of these compounds have been tabulated (24). Raman and infrared spectroscopy may also be

used to identify inorganic sulfites (25): most inorganic sulfites strongly absorb infrared radiation between 960 and 910 cm.$^{-1}$.

BISULFITE-SULFITE OXIDATION

Representation of sulfite oxidation as

$$SO_3^{2-} + {}^1/_2\,O_2 \rightarrow SO_4^{2-}$$

gives the essential empirical equation for the reaction from which thermodynamic calculations may be made. This is not sufficient, of course, to give an insight into the effect of other ions or molecules on the oxidation process. In systems of pharmaceutical interest, sulfite will be present as an antioxidant in combination with compounds which may retard or accelerate bisulfite oxidation. Rate of bisulfite oxidation in such multicomponent systems will determine the relative effectiveness of this inorganic antioxidant. Brief consideration of a probable mechanism of sulfite oxidation in the absence of catalysts or retarders is desirable for a more complete understanding of its antioxidant activity in complex systems. It is emphasized that the mechanism presented, while consistent with available experimental data, does not preclude other equally probable mechanistic interpretations.

The autoxidative process of homogeneous inorganic systems appears to involve acceptance of electrons by O_2 following interaction with an electron donor (26). The OH^- ion appears to be the preferred electron donor leading to the complex O_2H^- which interacts with the reducing agent. Generalized anionic autoxidation may be described briefly in the following scheme proposed by Abel (27)

$$O_2 + A^- \rightarrow AOO^-$$
$$AOO^- + X \rightarrow 2O^- + X^+ + A$$
$$A + X \rightarrow X^+ + A^-$$

The qualitative dependence of autoxidations on pH can be rationalized on the basis of hydroxyl ion participation; most autoxidations proceed more rapidly in basic than in acidic solutions. In neutral or acidic solutions where anion concentrations are too low to participate in electron donation to O_2 by interactions, electron transfer may be an important step, e.g., Fe^{3+} with SO_3^{2-} (37).

According to Abel's generalized scheme presented above, bisulfite ion oxidation could be described by the following reaction sequence

$$O_2 + OH^- \rightleftharpoons O_2H^-$$
$$O_2H^- + HSO_3^- \rightarrow 2O^- + HSO_3 + OH$$
$$HSO_3 + OH \rightarrow 2H^+ + SO_4^{2-}$$
$$\overline{HSO_3^- + O_2 + OH^- \rightarrow SO_4^{2-} + 2OH}$$

Considerably more information has been conveyed than in the empirical equation $SO_3^{2-} + {}^1/_2\,O_2 \rightarrow SO_4^{2-}$. However, recourse has been made to the interaction of strange structures (OH, HSO_3) which contain atoms with an unshared electron. These complexes of abnormal valency are called free radicals or simply radicals (28); the chemistry of free radicals in solutions is a relatively new and exciting area of study (29). The free radical bisulfite oxidation will be presented in a stepwise manner with an attempt made to introduce radical participants in a rationalized fashion.

Production of O_2^- and OH radicals resulting from the interaction between oxygen and water may be assumed to precede or initiate the oxidation process. The hydroxyl ion arises from water ionization

$$\text{(I)}\quad H_2O \rightleftharpoons H^+ + OH^-$$

The anion interacts with oxygen to form free radicals

$$\text{(II)}\quad OH^- + O_2 \rightleftharpoons OH + O_2^-\,(O_2H^-)$$

Additional radicals result from interaction with other anions

$$\text{(III)}\quad O_2 + O^{2-} \rightleftharpoons O^- + O_2^-\,(O_3^{2-})$$

These radicals may interact with H^+ (from I) as follows

$$\text{(IV)}\quad O^- + H^+ \rightleftharpoons OH$$
$$\text{(V)}\quad O^{2-} + H^+ \rightleftharpoons OH^-$$

Sulfite (SO_3^{2-}) oxidation may result from interaction with the OH radical

$$\text{(VI)}\quad SO_3^{2-} + OH \xrightarrow{k_1} SO_3^- + OH^-$$

Involvement of the HSO_3 radical as an intermediate has been validated by numerous investigators (30); it arises as follows

$$\text{(VII)}\quad SO_3^- + 2H^+\,(\text{from I}) \rightleftharpoons HSO_3 + H^+$$

and the overall reaction (VI + VII) is

$$SO_3^{2-} + 2H^+ + OH \rightarrow HSO_3 + H_2O$$

Interaction of sulfite ion with the O_2^- radical may also take place

$$\text{(VIII)}\quad SO_3^{2-} + O_2^- \xrightarrow{k_2} SO_3^- + 2O^-$$

The HSO_3 radical arises as in VII; the overall reaction may be written as

$$\text{(IX)}\quad SO_3^{2-} + O_2^- + 3H^+ \rightarrow HSO_3 + 2OH$$

Product of the oxidation, sulfate ions (SO_4^{2-}) is formed by the interaction of HSO_3 and OH radicals

$$\text{(X)}\quad OH + HSO_3 \rightarrow SO_4^{2-} + 2H^+$$

The ionization constants of sulfurous acid (Ka_1 1.7×10^{-2}, Ka_2 6.24×10^{-8}) fix the ratio of sulfite to bisulfite ion at any pH and unbuffered solutions prepared with sulfite salts will contain HSO_3^- ions as a consequence of sulfite hydrolysis

$$SO_3^{2-} + H_2O \rightleftharpoons HSO_3^- + OH^-$$

Bisulfite, HSO_3^-, oxidation also involves the HSO_3 radical as an intermediate; it may arise from the interaction of the ion with the OH radical

$$\text{(XI)} \quad HSO_3^- + OH \xrightarrow{k_3} HSO_3 + OH^-$$

the overall reaction being

$$\text{(XII)} \quad HSO_3^- + OH + H^+ \text{ (from I)} \rightarrow HSO_3 + H_2O$$

Interaction of bisulfite ion with O_2^- radical may be shown to yield the HSO_3 radical

$$\text{(XIII)} \quad HSO_3^- + O_2^- \xrightarrow{k_4} HSO_3 + 2O^-$$

the overall equation is

$$\text{(XIV)} \quad HSO_3^- + O_2^- + 2H^+ \text{ (from I)} \rightarrow HSO_3 + 2OH$$

Sulfate ions may be formed as in (X).

The combined rate expression representing total sulfite loss ($HSO_3^- + SO_3^{2-}$) occurring as described in expressions (VI), (VIII), (XI), (XIII) may be formulated

$$\text{(XV)} \quad \frac{-d(SO_3^{2-})}{dt} = (SO_3^{2-})\,[k_1(OH) + k_2(O_2^-)] + (HSO_3^-) \cdot [k_3(OH) + k_4(O_2^-)]$$

Evaluation of (XV) as a generalized equation leads to extremely unwieldy expressions due to the free radical terms. Abel's (31) extremely lucid and rigorous development included consideration of the radical initiation processes (II) and (III) with a steady state assumption for O^- and OH radical production. The reader is referred to Abel's detailed derivation which contains the explicit assumptions leading to the development of the following expression

$$\text{(XVI)} \quad \frac{-d(SO_3^{2-})}{dt} = \frac{g\,(HSO_3^-)}{\sqrt{H^+}}$$

where g includes combined free radical terms, etc.

Abel showed that Fuller and Crist's (32) critically determined experimental data could be evaluated with (XVI) and further pointed out that bisulfite rather than sulfite was the main species oxidized.

Thiosulfate, $S_2O_3^{2-}$, is a common contaminant in bisulfite solutions; it serves as a powerful catalyst for bisulfite oxidation. Thermodynamically unstable bisulfite yields thiosulfate (33) when stored in the absence of oxygen

$$\text{(XVII)} \quad 4HSO_3^- \rightarrow S_2O_6^{2-} + SO_4^{2-} + 2H_2O$$

the trithionate ion, $S_3O_6^{2-}$, is readily hydrolyzed to thiosulfate and sulfate

$$\text{(XVIII)} \quad S_3O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + 2H^+$$

The thiosulfate may participate in a reaction with a HSO_3 radical to yield more trithionate

$$\text{(XIX)} \quad S_2O_3^{2-} + HSO_3 \rightarrow S_3O_6^{2-}$$

or with a O_2^- radical

$$\text{(XX)} \quad SO_3^{2-} + S_2O_3^{2-} + O_2^- + 3H^+ \rightarrow S_3O_6^{2-} + OH + H_2O$$

or with a OH radical

$$\text{(XXI)} \quad SO_3^{2-} + S_2O_3^{2-} + 2OH + 2H^+ \rightarrow S_3O_6^{2-} + 2H_2O$$

The overall effect results in a marked catalysis. Chertkov (34) has shown the possibility of (XX) and (XXI) by thermodynamic calculations using free energy of formation data from Latimer (6). A sample calculation is shown to illustrate the usefulness of the method in assigning plausible mechanisms

$$SO_3^{2-} + S_2O_3^{2-} + O_2^- + 3H^+ \rightarrow S_3O_6^{2-} + OH + H_2O$$

$$(-116.1) + (-124) + (+13) + (0) \rightarrow (-229) + (+8.53) + (-56.69)$$

The total free energy change, ΔF^0, for (XX) is -50 Kcal. mole^{-1} and for (XXI) -119.3 Kcal. mole^{-1}, indicating that the reactions are theoretically possible.

Traces of copper ions act as powerful catalysts in sulfite oxidation. Various mechanisms have been proposed for their catalysis which in outline form is

$$\text{(XXII)} \quad 2\,(2Cu^{2+} + SO_3^{2-} + H_2O \rightarrow 2Cu^+ + SO_4^{2-} + 2H^+)$$

$$\text{(XXIII)} \quad 4Cu^+ + O_2 + 4H^+ \rightarrow 4Cu^{2+} + 2H_2O$$

$$\text{(XXIV)} \quad 2SO_3^{2-} + O_2 \rightarrow 2SO_4^{2-}$$

Fuller and Crist (32) assumed (XXII) to be the rate-determining sequence by formulating the catalytic effect proportional to the product of Cu^{2+} and SO_3^{2-}. This requires that the sequence (XXIII) be assumed to take place at a much greater rate than (XXIII); however, this leads to certain inconsistencies inasmuch as cuprous ion is known to be rate-determining in the analagous hydrogen peroxide-thiosulfate reaction (35). Abel (31) has advanced a strong case for (XXIII) as the rate-determining step and has developed a lucid, consistent mechanism to support this

viewpoint which involves a steady state assumption for the production of O_2^- and OH radicals and a time-dependent reaction with cuprous ion

$$\text{(XXV)} \quad \begin{aligned} Cu^+ + O_2^- &\rightarrow Cu^{2+} + 2O^- \\ Cu^+ + OH &\rightarrow Cu^{2+} + OH^- \end{aligned}$$

The mechanism, with explicit assumptions, leads to equations which will describe available experimental data.

Bisulfite Inhibitors.—The effectiveness of bisulfite as an antioxidant in typical pharmaceutical systems rests on the ease with which the compound is oxidized in comparison with the drug it is to protect. Substances which inhibit bisulfite oxidation may exert important effects on the overall stability of the product by decreasing the antioxidant effect of bisulfite. Bisulfite oxidation is strongly inhibited by a number of substances. The inhibition by mannitol (32, 36) is typical; it follows the empirical relationship

$$\text{(XXV)} \quad \frac{-d(SO_3^{2-})}{dt} = \frac{A\ k\ (SO_3^{2-})}{A + [\text{mannitol}]}$$

where A is a constant. Apparently many bisulfite inhibitors can be qualitatively described with (XXV) by substituting (inhibitor) for (mannitol) (37).

Aromatic anions often serve as effective bisulfite inhibitors: compounds such as aniline and the isomeric amino toluenes have been compared with hydroquinone in 0.1% concentrations as bisulfite inhibitors (38). Kinetic studies indicated *meta*-aminotoluene was about equal to hydroquinone as a bisulfite inhibitor. The mechanism of this inhibition has been postulated as involving formation of co-ordination compounds between the inhibitor and bisulfite. Stability of these bisulfite complexes or co-ordination compounds appears to be related to the dipole moment of the inhibitor.

Various phenolic compounds also tend to inhibit bisulfite oxidation; however, it is difficult to assess the quantitative relationships (39). A comparative study of the inhibiting action of phenols showed the following order of effectiveness: hydroquinone > pyrogallol > 2-naphthol > 1-naphthol > pyrocatechol > resorcinol > phenol > phloroglucinol.

BISULFITE IN FORMULATIONS

The use of bisulfite in formulations follows something of an empirical approach both in regard to its initial choice as an antioxidant and with regard to the amounts or concentrations employed. Although patent literature is generally a rich source of formulations employing bisulfite as an antioxidant (40), specific amounts are only rarely recommended. Many formulations contain from 0.01 to 1.0% of bisulfite or sulfite, but some few formulations have contained more or less than this. The best rule to follow is to use the minimum amount of bisulfite which will give the desired antioxidant action.

The calculation of minimum required bisulfite is best illustrated for the case of an ampul containing an autoxidizable drug which has been shown to be protected with, and not adversely affected by, sulfite. The sealed, glass ampul represents a unique system isolated from external environment: vapor or gas diffusion through the glass wall is almost nonexistent; a fixed amount of oxygen is present in the ampul dissolved in the vehicle or in the air head space above the liquid. Nitrogen sparging of ampuls or vacuum sealing will materially decrease oxygen content prior to sealing; however, this is not considered for this simplified example: 5 ml. of the liquid formulation is to be filled into an ampul and sealed with approximately 1 ml. of air head space above the liquid. Assume that all of the oxygen comes from air (23.14% by weight) in the head space. One milliliter of air, 25°, 760 mm. = 8.56×10^{-3} mM of O_2 = 17.10×10^{-3} mM or 1.768 mg. of sodium bisulfite (104.07 mg. mM^{-1}).

The same formulation packaged as a multiple-dose vial may require additional bisulfite due to the fact that an equal volume of air is injected to facilitate withdrawal of the dose. Thus, if five 1-ml. doses were contained in a multiple dose vial with 1 ml. air head space, available bisulfite might be overwhelmed by the oxygen from injected air with resultant autoxidative destruction of the labile drug. No hard and fast rules can be formulated since much depends on how quickly the remaining doses are utilized, etc. The problem of stabilizing oral dosage forms packed in glass is similar to that of the multiple-dose vial; each time a dose is removed, additional air (oxygen) is introduced into the system. Packaging bisulfite-stabilized formulations in certain plastic containers presents unusual difficuties if the plastic is gas-(oxygen) permeable; the constant diffusion of oxygen from the infinite atmospheric reservoir into the liquid represents an inexorable driving force for bisulfite oxidation. Packaging in glass or special dense plastic is the best answer to this problem.

Rubber-stoppered multiple-dose vials containing bisulfite solution may loose water at an unexpectedly high rate due to penetration of the rubber by bisulfite which changes the sorption characteristics of rubber (41). The reaction of rubber

caps or stoppers with bisulfite has been extensively studied (42). Precoating or soaking of stoppers with bisulfite solution can reduce bisulfite loss from solution (43, 44). Dry powders have been protected against deterioration from atmospheric oxygen by placing in the container envelopes containing pellets of sulfite which readily oxidize to sulfate in the presence of oxygen and having accelerators such as hydrated calcium or copper salts present (45).

The choice between bisulfite or pyrosulfite and sulfite for use in a formulation should generally be made on the basis of the final pH of the solution, and with regard to the available SO_2 content of the salt. Addition of sodium sulfite, Na_2SO_3, to an unbuffered drug solution of pH 3 to 5, results in the need for subsequent pH adjustment or, at best, a decrease in the buffer capacity of the formulation. It is better practice to use bisulfite or pyrosulfite in solution when these anions predominate by virtue of the final solution pH. Conversely, for an unbuffered solution of pH 8 one should use sulfite (SO_3^{2-}) salts.

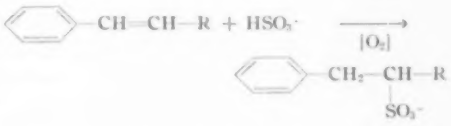
Determination of possible adverse effects on the formulation resulting from bisulfite inclusion may be accomplished by simply determining bisulfite content in the formulation when stored at high temperature under nonoxidative conditions (46, 47). The following represents a simple, useful procedure which should be followed with any new formulation which contains bisulfite, pyrosulfite, or sulfite as an antioxidant: Sparge the formulation with nitrogen and fill into hard glass ampuls. Sparge the ampuls with nitrogen and seal (or seal under vacuum). Place the ampuls in a thermostat and maintain temperature at 60 to 80°. Periodically withdraw samples and assay iodometrically for bisulfite content.

Obviously the procedure serves to identify only those formulations in which bisulfite participates as a reaction, i.e., bisulfite is consumed. Specific ion catalysis by bisulfite and other degradation processes require more elaborate stability studies and assays for active components. Isolation procedures and kinetics and mechanism studies which were employed in characterizing the epinephrine-bisulfite reaction (48, 49) may serve as a guide in evaluating other bisulfite-reactive drugs.

The solubility of the commonly employed bisulfite, pyrosulfite, and sulfite salts of group I-a elements is sufficiently high so that few difficulties arise on this account. However, the solubility of II-a salts such as calcium sulfite, $CaSO_3$, is lower than the corresponding alkali metal salts by several orders of magnitude. Obviously these salts are not generally added as such but if the formulation contains Ca^{2+} these will be formed by a metathetical reaction, e.g., $Ca^{2+}A^{2-} + Na_2SO_3 \rightarrow CaSO_3 \downarrow + Na_2A$ which results in precipitation of insoluble calcium sulfite. The SO_2 content of the various salts on a weight basis gives a means of evaluating relative reducing power

Salts	Formula	SO_2, %
Ammonium hydrogen sulfite (bisulfite)	NH_4HSO_3	64.64
Ammonium sulfite	$(NH_4)_2SO_3 \cdot H_2O$	47.75
Potassium hydrogen sulfite (bisulfite)	$KHSO_3$	53.32
Potassium pyrosulfite (metabisulfite)	$K_2S_2O_5$	57.6
Potassium sulfite	$K_2SO_3 \cdot 2H_2O$	32.97
Sodium hydrogen sulfite (bisulfite)	$NaHSO_3$	61.56
Sodium pyrosulfite (metabisulfite)	$Na_2S_2O_5$	67.39
Sodium sulfite	Na_2SO_3	50.82

Reaction of aldehydes or certain reactive ketones with bisulfite and the addition of bisulfite to the alkene linkage has been intensively studied (50, 51); bisulfite reactivity of drug molecules which contain these functional groups is generally recognized but probably warrants a brief discussion for sake of completeness. Bisulfite reacts rapidly with the alkene linkage to give high yields of sulfonic acids (52). The reaction very likely occurs by a free radical mechanism since addition is always contrary to the Markovnikov rule and appears to be dependent on the presence of oxygen



The following mechanism (53) appears to be consistent with the observed data

Initiation step:

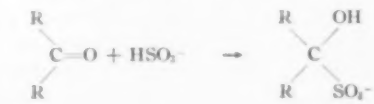
$$SO_3^{2-} + OH \rightarrow SO_3^- + OH^-$$

Propagation step:

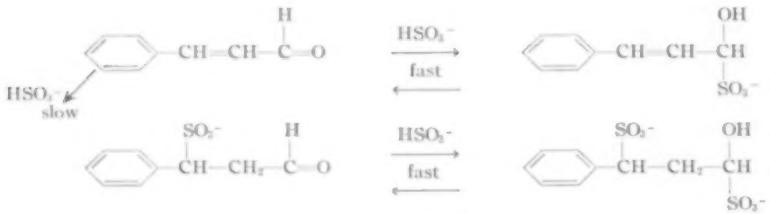
$$SO_3^- + R{-}CH{=}CH_2 \rightarrow R{-}CH{-}CH_2SO_3^-$$

$$HSO_3^- + R{-}CH{-}CH_2SO_3^- \rightarrow RCH_2CH_2SO_3^- + SO_3^-$$

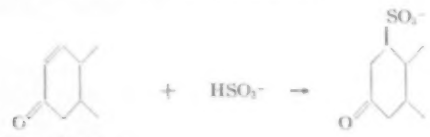
Hydroxysulfonic acids are readily formed in aqueous solution by the reaction of bisulfite with reactive carbonyl compounds (54)



Mechanism of the reaction appears to be complex (55); sulfite ion has been reported to be the reactive nucleophilic species rather than bisulfite ion. The reaction appears to be sensitive to steric hindrance: most aldehydes react but reaction with ketones is limited to methyl or cyclic ketones. Keto esters such as ethyl acetoacetate and ethyl pyruvate also yield bisulfite addition products and 1,2-diketones tend to form dihydroxysulfonates (56). Bisulfite reaction with α,β-unsaturated aldehydes or ketones (57) may yield either the normal 1,2-addition product (hydroxysulfonate) or a 1,4-addition product (sulfonic acid) or both products may be formed



Conjugate addition is the rule rather than exception with α,β-unsaturated ketones (52, 58). The 3-keto Δ^1 system of certain steroids appears to be much more reactive toward conjugate bisulfite addition than either the corresponding 3-keto Δ^4 or the 3-keto $\Delta^{1,4}$ compounds (59)



3-keto Δ^1 system

However, it should be noted that 3-keto $\Delta^{1,4}$ steroid such as prednisolone phosphate do undergo a reaction with bisulfite or pyrosulfite leading to infrared spectral changes which suggest presence of only one conjugated A ring double bond in the product (60).

The reactions of bisulfite with alkyl halides to yield alkyl sulfonic acids (61) and with aromatic nitro compounds to yield arylamino sulfonic acids (62) are less known; however, not many drug molecules containing these functions are susceptible to autoxidation.

The number of pharmaceutical formulations which have employed bisulfite as an antioxidant would make an impressive listing; however, the scope of its usefulness is best shown with selected examples. Inclusion of bisulfite into formulations containing dyes should always include careful observations of the fading tendency of the dye (42). Discoloration of dextrose solutions upon autoclaving may be prevented by the addition of bisulfite (63, 64). No difference was found in the incidence of thrombosis following infusion of discolored solutions and similar solutions in which discoloration was prevented by bisulfite (65). Discoloration of streptomycin and dihydrostreptomycin preparations may be prevented by the addition of bisulfite (42, 66). Procaine penicillin and other penicillin suspensions have been effectively stabilized against oxidative degradation by using 0.33% sodium bisulfite (67). The darkening of para-aminobenzoic acid solutions may be prevented by the addition of bisulfite to the formula (42). Discoloration of sodium aminosalicylate solutions may be decreased or prevented through the use of bisulfite antioxidants, but there is some evidence that bisulfite facilitated a nonoxidative degradation (68). Bisulfite has been reported to be the preferred antioxidant for steroid phosphate solutions (69); it is useful with other steroid preparations (70). Comparative studies with various antioxidants in sodium sulfadiazine formulations have shown bisulfite to be the most effective in preventing color formation (71, 72). Morphine solutions have been stabilized against color formation with bisulfite (73); however, there is some indication that morphine forms an addition product with bisulfite. Neomycin preparations show good color stability when bisulfite is included in the formulation (74). Stabilization of epinephrine-procaine solutions with various antioxidants demonstrated the superiority of bisulfite in preventing solution oxidation with attendant color formation (75). It has been shown that bisulfite reacts with epinephrine to form a colorless, inactive epinephrine sulfonate (46), thus pointing out the need for caution in the use of bisulfite in formulations without adequate studies to determine its effect on active ingredients. The reaction between bisulfite and epinephrine is not unique; sympathomimetic and other drugs which are *ortho-* or *para-* hydroxybenzyl alcohol derivatives will react with bisulfite to yield corresponding sulfonic acid derivatives which are usually inactive (47).

Riegelman and Fischer (76) have demonstrated that chelation of the catechol nucleus with borate results in marked stabilization of epinephrine against bisulfite attack.

TOXICITY

Sulfites have been extensively used in the food industry to prevent darkening of dried fruits and fruit juices in amounts from 1,500 to 2,000 p.p.m. (77); sulfite has also been applied to vegetables after blanching in similar amounts. The use of sulfites in foods is, of course, subject to government regulation (78). While this does not indicate explicit official approval for all formulations or products containing bisulfite, it does imply some degree of relative safety for the use of these antioxidants. Although the percentage of sulfite in foods is generally much smaller than in pharmaceutical formulations, it must be emphasized that vastly greater amounts are consumed in foods than in pharmaceutical products.

Chronic toxicity of a diet containing 0.1% sodium bisulfite, 615 p.p.m. as SO_2, was determined by long-term administration to rats in extensive experiments conducted by Fitzhugh, *et al.* (79). Observed toxicity appeared to be related, in part to thiamine destruction of the diet by the sulfite; however, biweekly thiamine injections did not prevent histopathological changes completely. Considerable merit may be given to Lockett and Natoff's suggestion (80) that the primary toxicity of a sulfited diet was related to the formation of sulfite interaction products rather than the well-known thiamine destruction (81). There is an abundance of chemical evidence for the formation of sulfite interaction products with saccharides (82) and the toxicity of these hydroxysulfonates has not been studied in detail. The carefully conducted experiments by Lockett and Natoff (80) indicated that the administration of sodium bisulfite, 750 p.p.m. SO_2, in the drinking water of rats is nontoxic. Their experiments were extended over a period of three years and involved three generations of rats. The weight of fluid drunk exceeded that of diet consumed by each generation of rats. The drinking fluid was prepared daily and assayed for SO_2 content to assure validity of the experiment, any significant autoxidation of the fluid to sulfate would have negated the toxicity study.

Acute toxicity, LD_{50}, of sodium bisulfite administered intravenously to rats is 115 mg./Kg. and the LD_{50} of sodium sulfite administered intravenously to the mouse is 175 mg./Kg. (83). Minimum lethal intraperitoneal dose of bisulfite for white mice weighing between 22 and 25 Gm. was 0.2 ml. of 0.6 *M* solution (84). In the same study, the minimum lethal i.v. dose for rabbits was 1.02 m*M* per Kg. It is interesting in regard to parenterally administered bisulfite to consider its effect in the anerobic carbohydrate metabolism of muscles. If sulfite is added to glycolyzing muscle extracts, the pyruvic acid is trapped in the formation of a bisulfite compound. This has a blocking effect on the mechanism for DPNH reoxidation (85) which results in dihydroxyacetone phosphate reduction (DPNH/glycerophosphate dehydrogenase) to α-glycerophosphate and formation of equal amounts of glycerophosphate and pyruvic acid from fructose 1,6-diphosphate. Oral acute toxicity in man appears not to be a significant problem if the observation of Lafontaine and Goblet (86) is correct that the vomiting reflex operates at a dose less than 3.5 mg./Kg. This dose level is interesting to consider in terms of usual doses of from 0.3 to 0.6 Gm. reported for sodium bisulfite when formerly employed in fermentative dyspepsia (87). The lower usual dose (0.3 Gm.) appears to exceed Lafontaine's vomiting reflex level for a 70-Kg. patient (245 mg.). No wonder it is no longer a popular treatment! The metabolic fate of orally ingested sulfite seems to follow the pattern for sulfate inasmuch as sulfites appear to be converted *in vivo* as well as *in vitro* to sulfate (88); sulfates are excreted principally in the urine as inorganic salts (90%) and as ethereal sulfates (10%).

External application of sulfite solutions for the treatment of parasitic infections in humans occasionally leads to mild dermatitis; however, these solutions are very much more concentrated than those in which bisulfite is present as an antioxidant. Similarly, hair waving lotions containing bisulfite as the active ingredient have caused dermatitis (89) but the very dilute (*ca.* 0.1%) bisulfite solutions have rarely been indicted as a cause of dermatitis. Dermal irritation caused by relatively concentrated bisulfite solutions has been studied with rabbits and pigs (84).

Ophthalmic preparations have utilized bisulfite, pyrosulfite, and sulfite salts as antioxidants, and tables of sodium chloride equivalents and freezing point data are available (83). At the isosmotic concentrations of sodium bisulfite (1.50%) no hemolysis of human erythrocytes occurs (90).

SUMMARY

Bisulfites, pyrosulfites, and sulfites have been widely employed for many years in pharmaceutical formulations on a purely empirical basis. Effectiveness of these compounds as pharmaceu-

tical antioxidants in most aqueous systems depends on their avidity for free radicals such as OH or simply on the ease with which they are oxidized in comparison with most autoxidizable drugs. Choice of which sulfurous acid species to employ depends on the equivalent SO_2 content of the salt and on the final pH of the product. Calculation of the minimum amounts of bisulfite required for antioxidant protection and a procedure for evaluating bisulfite reactivity of components in a formulation permits more rational use of bisulfite antioxidants. Sulfurous acid salts are exceptionally effective antioxidants; their usefulness may be increased by an awareness of their chemistry, oxidative mechanism, and reactivity toward certain drug molecules.

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Research Articles

Color Reaction of Alkaloids with Dichromate-Sulfuric Acid and Its Use for Quantitative Assay

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Using as reagents potassium dichromate and concentrated sulfuric acid, a rapid, simple method for the assay of alkaloids has been developed. Dichromate concentration, acid normality, and the presence of salts influence the intensity of the color developed. The green color produced absorbs maximally at 650 mμ, is stable for at least 72 hours, and is insoluble in benzene, chloroform, carbon tetrachloride, and other organic solvents. The method has been applied successfully to the study of the interaction of alkaloids with hydrocolloids using the equilibrium dialysis technique. Furthermore, it affords a ready means of assaying samples of isolated alkaloids of several classes obviating the extraction of the pigment and involves no expensive reagents or elaborate equipment.

INTEREST in this laboratory in the interaction of alkaloids with hydrocolloids necessitated the use of a rapid test for the assay of small quantities of alkaloids. Available general tests (1) involving color development with dyestuffs, the silicotungstic-titanium chloride, and Reinecke salt procedure all lack rapidity for routine work. According to Wertheim (2), "Many alkaloids develop strong colors when treated with concentrated sulfuric acid alone or with the acid and a trace of potassium dichromate." Yohimbine, an indole alkaloid, in contact with concentrated sulfuric acid and potassium dichromate develops a violet streak which changes to blue and then to green (3). The use of dichromate and dilute acid has been applied to the determination of pilocarpine but involves an extraction step (4–6).

A cursory survey revealed that in the presence of these reagents, several alkaloids of varying classes and the so-called xanthine alkaloids, caffeine, theobromine, and theophylline, gave an intense green color with an absorption maximum at 650 mμ. With adequate control, the intensity of the color developed is proportional to the amount of alkaloid present.

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This communication reports on the conditions necessary for reproducible, quantitative assay. Several factors such as the influence of dichromate concentration, acid normality, color stability, and the influence of added salts were investigated. Proposals are submitted for assaying the alkaloids when alcohol is present. The method has been applied successfully to a study of the interaction of alkaloids with hydrocolloids by use of the equilibrium dialysis technique.

EXPERIMENTAL

Materials.—Alkaloids.—These are listed in Table I; concentrated sulfuric acid, reagent grade; potassium dichromate solution, 5% in distilled water; ethyl alcohol and methyl alcohol, absolute. Inorganic salts, organic salts, and other chemicals.—These are listed in Table II. They were all of U.S.P. or C.P. grade and were used without further purification.

Equipment.—Constant temperature water bath; Coleman Universal spectrophotometer, model 14; 30-ml Pyrex, glass-stoppered test tubes; 50-ml buret.

Procedure.—For all preliminary experiments and for investigating the influence of variables on the reaction, quinine hydrochloride only was used.

A 50 μM/ml. stock solution of quinine hydrochloride in distilled water was prepared and from this, solutions containing 1.25–25.00 μM/ml. were made up by dilution with distilled water. One milliliter from each of these latter solutions was placed in consecutive Pyrex, ground-glass-stoppered test tubes; 1 ml. of the 5% potassium dichromate solution was added, and the tubes incubated for 5 minutes in a constant temperature water bath at 30°. At the end of this period, 8 ml. of concentrated sulfuric acid was added to each tube, the tubes swirled gently to mix the contents well, and then placed in an ice water bath for 20 minutes. Zero concentration consisted of a tube containing 1 ml. of the dichromate and 1 ml. of distilled water and similarly treated. The color developed was measured at 650 mμ with a Coleman Universal spectrophotometer, model 14, using distilled water as the blank. The wavelength of maximum absorption (650 mμ) was determined by measuring the absorbance of solutions containing varying concentrations of the alkaloids at intervals over the wavelength range shown in Fig. 1 and plotting the absorbance as a function of the wavelength.

In adapting the method to experimental systems, the general procedure is the same, and the alkaloid concentration may be calculated from a standard curve.

Influence of Variables on the Reaction.—In order to evaluate the influence of several variables on the interaction, a constant amount of quinine hydrochloride (final concentration, 2.0 μM/ml.) was treated and the color developed as outlined in the general procedure.

The influence of dichromate concentration was ascertained by varying the added volume of the dichromate solution between 0.0–1.0 ml. Similarly, the effect of the final normality of the sulfuric acid was established by varying the added volume between 0.0–8.0 ml. so as to attain final normalities of 7.2 to 32.4.

The intensity of the color, developed according to the general procedure and measured at time intervals of 30 minutes to 72 hours, served as an index of color stability. Distilled water and sulfuric acid of 9.0–36 N used to dilute the color after it was developed, gave results reflecting the influence of the nature of diluents.

Table I.—Quantitative Response of Several Alkaloids and Ethyl Alcohol in the Dichromate-Sulfuric Acid Method[a]

Final Concentration μM/ml.	Absorbance at 650 mμ								
	Quinine HCl	Hydrastine HCl	Caffeine	Lobeline Sulfate	Tubocurarine Chloride	Pilocarpine HCl	Sparteine Sulfate	Yohimbine HCl	Hyoscyamine HCl
0.000	0.140	0.140	0.141	0.139	0.140	0.138	0.141	0.141	0.138
0.125	0.045	...	...	...	0.120	...	...	...	...
0.250	0.081	0.140	...	0.133	0.251	0.082	0.074	0.144	0.107
0.375	...	...	...	...	0.380	...	...	...	...
0.500	0.160	0.292	0.049	0.271	0.510	0.162	0.159	0.279	0.217
0.750	...	...	...	0.406	0.770	...	...	0.419	...
1.000	0.320	0.593	0.101	0.540	...	0.330	0.315	0.552	0.442
1.500	0.480	0.870	...	...	...	0.550	0.475	...	0.666
2.000	0.641	...	0.201	...	...	0.662	0.629	...	...
3.000	...	...	0.309	...	...	...	...	...	...
4.000	...	...	0.412	...	...	...	...	...	...
				Ethyl Alcohol					
0.000	...	...	...	0.142	...	...	...	...	...
2.000	...	...	...	0.063	...	...	...	...	...
4.000	...	...	...	0.128	...	...	...	...	...
6.000	...	...	...	0.193	...	...	...	...	...
8.000	...	...	...	0.253	...	...	...	...	...
10.000	...	...	...	0.318	...	...	...	...	...
12.000	...	...	...	0.279	...	...	...	...	...
14.000	...	...	...	0.443	...	...	...	...	...
16.000	...	...	...	0.508	...	...	...	...	...

[a] All values corrected, i.e., A of System − A for zero concentration; listed in top half of table. All responses were good (± 1.0–±2%) except hydrastine hydrochloride which was fair (±5%).

TABLE II.—INFLUENCE OF ADDED SALTS AND UREA ON COLOR INTENSITY

Salt Added	Limiting Concentration (M)
Lithium chloride	2×10^{-3}
Sodium chloride	2×10^{-3}
Potassium iodide	5×10^{-4}
Potassium chloride	1×10^{-3}
Ammonium chloride	1×10^{-3}
Potassium nitrate	2×10^{-3}
Sodium phosphate	1×10^{-4}
Sodium dihydrogen phosphate	2×10^{-3}
Magnesium chloride	2×10^{-3}
Strontium chloride	2×10^{-3}
Manganese chloride	1×10^{-3}
Barium chloride	2×10^{-3}
Mercuric chloride	2×10^{-3}
Sodium carbonate	2×10^{-3}
Calcium chloride	1×10^{-3}
Sodium thiosulfate	1×10^{-4}
Manganous sulfate	2×10^{-4}
Ferric chloride	2×10^{-3}
Dibasic ammonium citrate	2×10^{-4}
Sodium benzoate	1×10^{-2}
Potassium tartrate	2×10^{-4}
Sodium salicylate	1×10^{-4}
Potassium acid phthalate	2×10^{-4}
Sodium acetate	2×10^{-2}
Urea	2×10^{-2}

The influence of several salts or other compounds, many of which may be incorporated into pharmaceutical preparations containing alkaloids, was assessed by adding increasing amounts to a constant quantity of the alkaloid, developing the color according to the general procedure, and comparing its intensity to that of a control system. That concentration of added salt or other compound above which a deviation from the control of greater than 1% transmission is produced is, for convenience, termed the "limiting concentration."

Alkaloidal Spectrum of the Method.—Since several alkaloids were to be used in the equilibrium dialysis studies, it was necessary to ascertain whether the characteristic green color would be given by alkaloids of several classes. Small amounts of the alkaloids were added to Pyrex, glass-stoppered test tubes. One milliliter of the dichromate solution and 8 ml. of concentrated sulfuric acid were then added, as in the general procedure. Each tube was then observed to see if the typical green color developed and the absorption spectrum checked, as outlined under the general procedure.

Quantitative Nature of the Test.—After the influence of the several variables was established, 1 ml. of quinine hydrochloride solution, containing amounts to give the final concentrations listed in Table I, were placed in consecutive test tubes and the color developed and measured according to the general procedure. Quantitative relationships were found to exist for quinine hydrochloride and several other representative alkaloids, as shown in Fig. 2 and Table I. The zero concentration (dichromate plus sulfuric acid) readings, $A = 0.138$–0.141, were subtracted from all measured values to give the corrected data recorded in Table I.

Influence of Ethyl Alcohol.—Many alkaloids are only slightly soluble in water but readily soluble in ethyl alcohol. However, alcohol responds to the test (Table I) and must either be removed or its influence be fully accounted for. If the alkaloid is soluble in water to even a slight extent, the alcohol can be removed by heating the mixture in a boiling water bath. The alkaloid can then be dissolved in water and determined according to the standard procedure.

Where the alkaloid is water-insoluble, the alcohol-free residue can be redissolved in a "known amount"

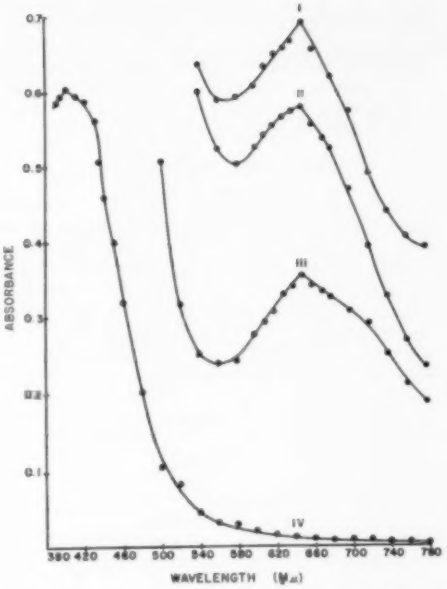

Fig. 1.—Absorption spectra of colors developed by alkaloids and caffeine in the dichromate-sulfuric acid method. All plotted values are uncorrected. I, Caffeine (5.375 μM/ml.); II, quinine hydrochloride (1.375 μM/ml.); III, hyoscyamine hydrochloride (0.5 μM/ml.); IV, dichromate plus sulfuric acid (0.05 ml. of 5%) potassium dichromate).

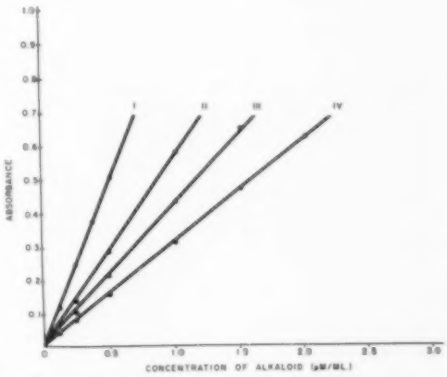

Fig. 2.—Quantitative response of alkaloids in the dichromate-sulfuric acid method. All values are corrected for zero readings. I, Tubocurarine hydrochloride; II, hydrastine hydrochloride; III, hyoscyamine hydrochloride; IV, quinine hydrochloride.

of alcohol. A standard curve for the alkaloid established with the use of the same "known amount" of alcohol can then be employed to assay the alkaloid being investigated. The validity of this procedure depends on the similar absorption spectrum and quantitative response of ethyl alcohol. Methyl alcohol responds similarly.

DISCUSSION AND SUMMARY

In the proposed method, the amount of potassium dichromate added has a profound influence on the final absorbance (Table III). As the added amount increases, the absorbance increases but no strict maximum was observed. At very low levels of dichromate, the intensity of the green color is drastically reduced due to incompletion of the reaction. At high concentrations, absorption by the excess dichromate apparently contributes to the absorbance. To assure completion of the reaction and reproducible results, an excess of dichromate should be present. Under the prescribed conditions, 1 ml. of the 5% solution is sufficient.

Table III.—Influence of Amount of Dichromate Added

Amount of Potassium Dichromate, ml.	Absorbance at 650 mμ	Color Produced
0.0	0.132	Yellow
0.1	0.134	Yellow
0.2	0.214	Greenish-yellow
0.4	0.398	Dark green
0.6	0.498	Dark green
0.8	0.602	Green
1.0	0.781	Green

Acid normality is of great importance and reproducibility can be attained only if the sulfuric acid has a final normality of 24.8 or greater.

The "heat of dilution" is sufficiently intense to effect rapid and complete reduction of the dichromate ion. Hydrochloric acid gives comparable results after 60 minutes at 30°, but all other acids tested will not suffice. The sulfuric acid can be added to the alkaloid-dichromate system after any desired time interval since this time lapse has no important influence on the final color intensity.

Twenty-five alkaloids belonging to the pyridine, quinoline, isoquinoline, quinolizidine, tropane, indole, and imidazole classes, as well as caffeine, theobromine, theophylline, and colchicine, gave the characteristic green color. Where rapidity is essential in assaying isolated samples or pure solutions of such alkaloids, the test will be of advantage since it obviates the extraction of the pigment as is necessary in the dye methods (1) and the method for pilocarpine (4). In addition, it involves no expensive reagents or elaborate equipment.

The orange-yellow precipitate formed from the reaction of the alkaloid with potassium dichromate in the case of pilocarpine, has been referred to as pilocarpine dichromate (5, 6). A similar reaction in the dichromate method for the determination of piperazine allegedly results in a precipitate of the formula

$$C_4H_{10}N_2 \cdot H_2Cr_2O_7 \text{ or } C_4H_8N_2 \cdot Cr_2O_7 \text{ (7)}$$

The green color formed is a result of an oxidation-reduction reaction (8, 9) in which the acidity is highly important. The change from orange-yellow to green is due to the formation of Cr^{+++}, according to the following half reaction

$$Cr_2O_7^{=} + 14H^{+} + 6e \rightleftarrows 2Cr^{+++} + 7H_2O$$

The pigment formed is different from that obtained in the test for pilocarpine (5) and is insoluble in benzene, chloroform, and other organic solvents.

Alcohol, the solvent of choice for many water-insoluble alkaloids, gives the characteristic color. Removal can be readily accomplished by heating. Where it must be present, an alcohol-alkaloid standard curve can be applied. Such an approach is based on the experimental evidence that, in the proposed test, alcohol responds in a quantitative manner (Table I).

Interference by salts, both inorganic and organic, must be noted. Apart from competitive interactions, their role in the oxidation-reduction can be significant and the limiting concentrations must be adhered to since, above these levels, serious deviations in the final color density occurs. The disturbing effects of inorganic ions have been noted and discussed by Walkley (9) in the application of the method to the analysis of organic carbon in soils.

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Complexation of Organic Substances in Aqueous Solution by Hydroxyaromatic Acids and Their Salts

Relative Contributions of Several Factors to the Overall Effect

By TAKERU HIGUCHI and ARGE DRUBULIS

Apparent stability constants in water of approximately 100 molecular complexes formed between aromatic acids hydroxylated to varying degrees and their salts, on one hand, and four organic compounds, theophylline, hydrocortisone, prednisolone, and phenacetin, on the other, are reported. The data show that introduction of —OH into the first group, particularly *ortho* to a carboxylate grouping, produced marked enhancement in binding. The relative magnitude of the constants suggest that neither the squeezing out force nor hydrogen bonding is significantly responsible in most instances for the observed effects.

THE PRESENT communication is concerned with the results of experimental studies designed to obtain further information on the nature of physical interaction responsible for formation of organic molecular complexes in dilute aqueous solutions. The relative roles of such factors as hydrophobic binding, hydrogen bonding, donor-acceptor forces, etc., have been considered. Data have been obtained specifically on the complexing behavior, as reflected in their solubilizing action, of various hydroxybenzoic acids, corresponding benzoates, several hydroxynaphthoates, and related compounds for theophylline, hydrocortisone, prednisolone, and phenacetin. The latter group was selected to provide a spectrum of compounds. Experimental techniques and interpretive approaches employed follow our earlier reports in this area

Information of this nature is of biological and pharmaceutical interest from two standpoints, (*a*) actions of drugs, hormones, enzymes, etc., are believed to be mediated by formation of complexes in aqueous environment, and (*b*) thermodynamic activities and solubilities of physiologically potent chemicals can be modified by complex formation.

EXPERIMENTAL

Reagents

All organic compounds used in these studies were purified prior to use by recrystallization from water or water-ethanol mixtures.

Procedure

Solubility Studies.—An amount of material to be tested equivalent to at least three times its normal solubility was weighed into 15-ml. vials containing varying amounts of complexing agent (acid or salt) and 10 ml. of distilled water. When the complexing agent was an acid, the water was replaced by an equivalent amount of 0.005 *M* sulfuric acid solution to decrease the ionization of the complexing agent. When the salt of an acid was used as the complexing agent, aliquots from a stock solution were added from a buret into the vials and the necessary volume of distilled water was added.

Each system was equilibrated by rotation for twenty-four hours for systems involving theophylline or phenacetin, and for forty-eight hours for those containing the steroids. This equilibration was effected in a thermostated water bath at a temperature of 30.0 ± 0.1°. The respective equilibration times were evaluated previously as sufficient time for each of the substrates.

Following equilibration, appropriate aliquots of clear solution were removed and analyzed for the material solubilized.

Analysis.—The aliquots were extracted with chloroform from a 0.5 *M* aqueous sodium bicarbonate solution. For theophylline, the extracting solvent was composed of three parts chloroform to one part isopropyl alcohol.

Analysis for theophylline or phenacetin was made directly in the chloroformic solutions. Analysis for the steroids was carried out in ethanolic solution. In these instances, the chloroform was evaporated with the use of mild heat and the substrate residue dissolved in 95% ethanol.

In all cases the spectral characteristics of the analyzed component, as determined previously in the proper solvents, were used as standards. The wavelengths chosen for spectral analyses were: theophylline, 275 mμ; hydrocortisone, 242 mμ; prednisolone, 245 mμ; and phenacetin, 249 mμ.

RESULTS

The complexing tendencies of the several free acids and the sodium salts of these aromatic and hydroxyaromatic acids, as reflected in their respective solubilizing power for theophylline, hydrocortisone, prednisolone, and phenacetin, are presented in Tables I and II. For the majority of the cases, the results are listed as first-order interaction constants (K_1), since the phase diagrams describing those systems indicated that the amount of material solubilized increased generally linearly with the

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TABLE I.—APPARENT STABILITY CONSTANTS[a] (L/M) FOR INTERACTIONS OF SOME MONO- AND DIHYDROXYBENZOIC ACIDS AND SALTS

Compound		Theophylline	Hydrocortisone	Prednisolone	Phenacetin
Benzoic	Acid	[c]	12	11	2.6
	Salt	5.3	2.9	3.7	1.0
o-Hydroxybenzoic	Acid	[c]	12	10	5.4
	Salt	20	7.2	7.3	1.9
m-Hydroxybenzoic	Acid	[c]	13	12	3.9
	Salt	11	4.9	6.1	1.1
p-Hydroxybenzoic	Acid	[c]	10[b]	11[b]	4.0
	Salt	12	4.1	5	1.2
2,4-Dihydroxybenzoic	Acid	[c]	12[b]	15	4.5
	Salt	58	13	12	2.4
2,5-Dihydroxybenzoic	Acid		16	15[b]	4.6
	Salt	43[b]	10[b]	12	2.4
2,6-Dihydroxybenzoic	Acid	[c]	12[b]	13	4.5
	Salt	300	13[b]	10[b]	3.3
3,4-Dihydroxybenzoic	Acid	[c]	12	14[b]	4.3
	Salt	14	6.8	6.5	1.5
3,5-Dihydroxybenzoic	Acid	[c]	18[b]	17[b]	4.9
	Salt	20	6.0[b]	6.5	2.0
3,4,5-Trihydroxybenzoic	Acid	[c]	19[b]	17[b]	5.3
	Salt	22[b]	7.3	6.2[b]	2.1

[a] Calculated by use of Eq. 1. [b] Best fit single linear constant describing interactions exhibiting curvature. [c] The interactions of these acids with theophylline were not studied since these compounds gave insoluble complexes.

TABLE II.—APPARENT STABILITY CONSTANTS[a] (L/M) FOR INTERACTIONS OF SOME NAPHTHOATE AND HYDROXYNAPHTHOATE IONS

Compound	Theophylline	Hydrocortisone	Prednisolone	Phenacetin
1-Naphthoate	38	8.2[c]	8.0[c]	2.5
2-Naphthoate	1:1.2[b]	19[c]	20[c]	5.8[c]
2-Hydroxy-1-naphthoate	1:1.4[b]	21[c]	23[c]	6.6[c]
1-Hydroxy-2-naphthoate	1:1.7[b]	35[c]	35[c]	7.0[c]
3-Hydroxy-2-naphthoate	1:1.6[b]	32	39	6.3

[a] Calculated by use of Eq. 1. [b] Stoichiometric ratios (salt : theophylline). [c] Best fit single linear constant describing interactions exhibiting some curvature.

amount of aromatic compound present, in accordance with the relationship

$$A + B \rightleftarrows AB$$
$$\Downarrow$$
$$[B]_{solid}$$

$$K_1 = \frac{[AB]}{[A]\ [B]} \qquad \text{(Eq. 1)}$$

where A is the acid or its salt, B is the substance solubilized, and AB is the complex formed.

A typical phase diagram exhibiting this linearity is that for the interaction of theophylline with sodium 1-naphthoate shown in Fig. 1. The stability constant can be calculated from such a diagram if any observed increase in solubility is assumed to be due to complex formation (1). Because of the nature of the experimental method employed this technique cannot, unfortunately, give any information as to the dependency of the complexing reaction on the concentration of B.

In a number of instances the dependency did not appear to be completely linear, the plots having an upward curvature. The solubilization of prednisolone by sodium 2-hydroxy-1-naphthoate, as shown in Fig. 2, for example, seems to be best described by a combination of more than one equilibrium occurring in solution at the same time. Although a better fit for such cases can be obtained by a combination of linear and quadratic terms, the data do not warrant such a refined treatment. For this reason the values for the best fit linear constant (K_1) (best fit linear constant, describing interactions exhibiting curvature) are given in these instances.

The values describing the binding behavior of some of the naphthoates with theophylline require additional explanation. Since, for these systems, one molecule of the naphthoate ion added usually brought more than one molecule of the xanthine into solution, as shown in Fig. 3, it is evident that the constant K_1, as defined above, is meaningless. The observations strongly support the suggestion that two or more theophylline molecules may interact with individual naphthoate or hydroxy-naphthoate ions. Since the experimental determinations were carried out at constant theophylline activity, the dependency of the complex formation on theophylline could not, of course, be established. Since these systems appear to be more or less linearly dependent on the concentration of naphthoate ion added, the results for these interactions are given as the molar ratio

$$\frac{\text{naphthoate added}}{\text{theophylline solubilized}}$$

It is possible that complexes of similar higher order dependency, relative to the solubilized species, may exist in systems other than those specifically cited in the preceding paragraph. The results are listed, nevertheless, in terms of interactions of first-order dependency since this probably is the most significant interaction occurring in these dilute systems.

DISCUSSION

As indicated in the data given in Tables I and II, aromatic acids and their salts appear to have definite affinity for the four organic compounds,

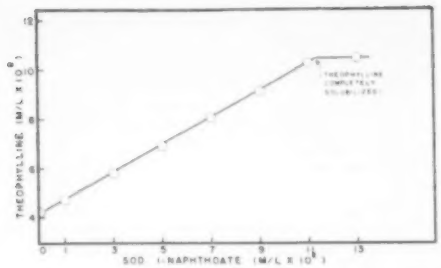

Fig. 1.—Plot showing the solubilization of theophylline by sodium 1-naphthoate exhibiting first-order dependency of complex formation on the salt concentration.

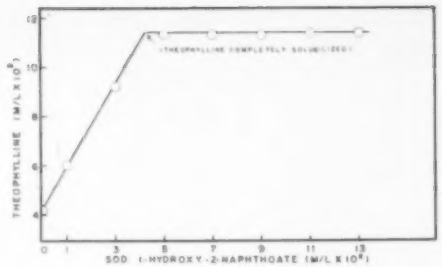

Fig. 3.—Plot showing the solubilization of theophylline by sodium 1-hydroxy-2-naphthoate exhibiting stoichiometric ratio greater than 1:1.

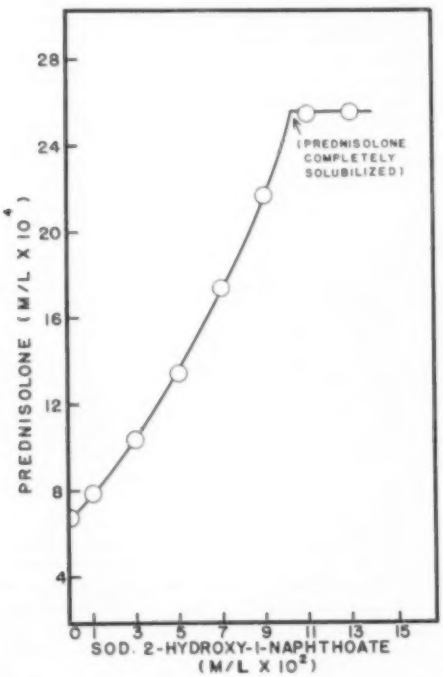

Fig. 2.—Plot showing the solubilization of prednisolone by sodium 2-hydroxy-1-naphthoate exhibiting mixed order dependencies of complex formation on the salt concentration.

The calculated apparent stability constants range from 1.0 L./mole for phenacetin and benzoate to 39 L./mole for prednisolone-3-hydroxy-2-naphthoate systems. For low concentrations of the complexed material this would mean that 0.1 molar concentration of the complexing agent would render roughly 10% of the first and 80% of the second system into their respective complexed species.

If we were to look only at the phenacetin systems, the weakness of the solubilization effects produced by the carboxylate salts hardly seems to justify attributing them to complex formation. Yet it is of interest to note that these largely parallel the behavior of the theophylline systems: a weak binder for phenacetin is a relatively weak binder for theophylline, and a strong binder for one is also a strong binder for the other. The binding constants for the xanthine are, on the other hand, generally so large that there is very little doubt that formal formation of new molecular species is involved in the solubilization process.

The same relative order of solubilizing action produced by the salt solutions appears to hold not only for theophylline and phenacetin but also for the two steroids, with only a few minor exceptions. This was somewhat surprising in view of the wide structural differences among the three classes of substrate compounds used. The relationship holds best with the two steroids since these structures are very similar, but the tendency is definitely evident even for the other systems. These results tend to suggest that much of the binding behavior exhibited in these instances, by these aromatic and hydroxy-aromatic compounds, was largely of a nonspecific nature as opposed to a behavior where more definite "lock and key" can exist between the components of a complex (2).

This lack of specificity does not, however, appear to extend to the acids. Salicylic acid, for example, appears to complex twice as much with phenacetin as does benzoic (5.4 L./mole *vs.* 2.6 L./mole), whereas with respect to the steroids, the activities of the two acids are essentially the same.

Interactions of Acid *vs.* Salt Forms.—In instances where both the free acid and its sodium salts were studied, the acid proved to be the stronger binder in every case. It is possible that this effect is a consequence of the lower hydrophilicity or the greater electrophilicity of the acid. With salt, the ions in solution are probably associated with several molecules of water situated near the carboxylate group. The directing effect of the formal charge on this group can influence the movement and the freedom of the water molecules, possibly resulting in the formation of ice-like structures. It is possible that the presence of such structures may hinder the association involving the aromatic ring materially. The negative charge on the ionized species may, on the other hand, be responsible for sufficient reduction of the electrophilicity of these compounds to reduce their affinity for electron donating agents.

No data are reported for interactions of free acids with theophylline as these systems led, in nearly all cases, to formation of insoluble complex species.

The solubility of the molecular compounds found were usually so low that no reliable stability constants could be determined (3).

Hydroxy- and Methoxybenzoates.—The relative complexing abilities of salicylic acid, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acids, together with those of their salts, are shown in Table I. The introduction of an hydroxyl group into the ring markedly enhanced the binding tendencies of the carboxylate forms. With the *ortho* substituent, the effect is essentially doubling of the observed stability constants over those found for the simple benzoate. The enhancement for the *meta* and *para* isomers is somewhat less but still significant, being roughly one-half of that of the *ortho*. The binding tendencies of the free acids do not appear to be markedly influenced by the addition of an hydroxyl group except with phenacetin. With the latter, the *ortho* substitution again produced doubling of the constant over that with benzoic acid.

The reason for the apparent greater complexing efficacy of hydroxybenzoates, particularly salicylate, over that of benzoate is not altogether clear at the moment. Certainly if the driving force behind these interactions were largely due to the squeezing out effect (1) or hydrophobic bonding, the introduction of an —OH grouping would not be expected *a priori* to increase the associative tendencies. A possible explanation for the observed nonspecific increase in binding may be in the changes produced in the relative electrophilicity of these compounds. The four compounds against which these aromatic compounds were tested are largely electron donors in nature. If the major effect in going, for example, from benzoate to salicylate is in the relative affinity for the π-electrons of the donor molecules, the observed results may be rationalized.

Possible participation in a molecularly dispersed state by dipolar or, specifically, hydrogen bonds in promoting molecular association between these hydroxy compounds and xanthine, steroids, and phenacetin appeared to be small. For one thing, such interactions would be in direct competition with the water molecules, the net gain in energy being small. Also, more significantly, although the position and presence of the hydroxyl groups markedly enhanced the intensity of interaction, the effect was largely nonspecific with respect to the solubilized component. There could not be direct fits, since the relative order of interaction did not appear to differ markedly for each of the substances.

To shed additional light on the nature of the hydroxyl contribution, the binding tendencies of *ortho*- and *meta*-methoxybenzoates with respect to theophylline were determined. The first-order constant found for *o*-methoxybenzoate was 5.3 L./mole and that for *m*-methoxybenzoate was 17 L./mole. The marked *ortho* effect noted for the phenolic benzoate thus appears to be absent for the methoxy system. For the *meta* isomer, the replacement of the proton seems to enhance the binding tendency. These results, again, seem to preclude hydrogen bonding as a major factor in these interactions.

Interactions of Di- and Trihydroxybenzoates.—Comparison of the results obtained for the interactions of the dihydroxybenzoates with those of the monohydroxybenzoates indicates that the effect of the additional hydroxyl group further intensified these interactions. Again, to substantiate the thought that this was not due to hydrogen bonding, the binding tendency of 3,4,5-trimethoxybenzoate with respect to theophylline was determined. The first-order constant for this interaction was 23 L./mole; this compares well to that obtained for the trihydroxy compound (22 L./mole). Thus, it is further evident that the presence of the phenolic protons was not of major importance in these interactions.

In general, the total effect that additional groups may have on the binding ability of a bifunctional or trifunctional compound may conceivably be of two types. It is possible that the added groups may enhance the strength of the initial complex maintaining essentially the same structure, or the new groups may lead to the formation of another complex species independent of the initial complex. In the former case, the energies of interaction are, in a sense, additive, while in the latter case, the extents of the interactions are statistically additive.

If the effect of the two hydroxy groups is such that the interaction energies are additive, then it can be easily seen that the interaction constant (K) for the dihydroxy isomer may be approximately related to the interaction constants for the monohydroxy compounds in the following manner

$$K_D = \frac{K_A}{K_C} \times K_B \qquad \text{(Eq. 2)}$$

K_D is the interaction constant for the dihydroxybenzoate, where K_A and K_B are the interaction constants for the respective monohydroxybenzoates, and K_C is the interaction consant for benzoate alone. The assumption made here, of course, is that the two hydroxyl groups do not interact appreciably with each other.

If, on the other hand, the hydroxyl groups do not make use of the same structure in exerting their binding action, then it is apparent that

$$K_D = K_A + K_B - K_C \qquad \text{(Eq. 3)}$$

The data obtained in this study with the dihydroxybenzoates suggest that in these associations the hydroxyl groups generally appear to reinforce the initial binding of the benzoate nucleus and the interaction constants for these compounds follow the relationship expressed by Eq. 2. A comparison between the experimentally observed values and the calculated values is presented in Table III. It is evident that these values agree for each of the substrates in a very approximate sense. In view of the obvious assumptions implicit in such treatment, these data should be considered only as qualitative indications.

Interaction of Naphthoates.—Expansion of the ring system from a benzenoid to the naphthalene structure may be expected to lead to increased complexing tendencies on at least two counts. The greater hydrophobic surface of the flat naphthalene ring surely must contribute more substantially to the "squeezing out" tendency. Also, the relative electron affinity of the two systems must be sufficiently different to produce some changes. Data show, in any case, very strong binding tendencies by these compounds.

As is evident from Table II, β-naphthoate was a markedly stronger binder than its isomer, α-naphthoate. Since this relative difference in bind-

TABLE III.—COMPARISON OF EXPERIMENTAL (*E*) AND CALCULATED[a] (*C*) INTERACTIONS CONSTANTS FOR DIHYDROXYBENZOATES

	Theophylline		Hydrocortisone		Prednisolone		Phenacetin	
	E	C	E	C	E	C	E	C
2,4-Dihydroxybenzoate	56	45	13	10	12	10	2.4	2.3
2,5-Dihydroxybenzoate	43	42	10	12	12	12	2.4	2.1
2,6-Dihydroxybenzoate	300	75	13	18	11	14	3.3	3.6
3,4-Dihydroxybenzoate	14	25	6.8	6.9	6.4	8.4	1.5	1.3
3,5-Dihydroxybenzoate	20	23	5.6	8.3	6.5	10	2.0	1.2

[a] Obtained by use of Eq. 2.

ing was true for all four systems, it is somewhat unlikely that the effect could be attributed to specific steric interactions. There is a possibility, however, that steric interference between the carboxylate group and the second ring may confer flatter configuration to the β-isomer; this may be generally more conducive to binding. The observed greater interactions, on the other hand, may again be attributed to the possibly greater electron affinity of the β-isomer, although it would be difficult to explain why this would be the case.

The results for the few hydroxynaphthoates studied indicate that the added effect due to the presence of a hydroxyl group on the naphthalene ring was approximately the same as it was for the presence of this group on the benzene ring. This observation tends to suggest that the factors responsible for the increased complexation of the monohydroxybenzoates over benzoate apparently hold for these naphthoate systems as well. Again, it is difficult to make any conclusions which would be more than speculative, particularly in view of the limited systems studied.

Considerations from the Standpoint of the Material Solubilized.—The degree of solubilization achieved is, as expected, strongly dependent on the nature of the compound. The data of Tables I and II reveal that the most nucleophilic compound, theophylline, was the strongest binder in all instances. Previous studies (1, 2) have shown that the xanthines generally are strong binders of electron acceptors. It is probable that the contribution from this type of binding, reinforced by other factors, is also important in the interactions with these aromatic and hydroxyaromatic compounds.

One might expect from the type of structures involved that hydrophobic associations would be responsible, on the other hand, for the binding tendencies of the steroids. Contributions from such binding alone could not, however, explain the enhancing ability of phenolic groupings. Consequently, it is felt that π-electrons in the keto group and in the double bond(s) of the A ring must participate to some extent in these interactions.

The relatively low associative tendency of phenacetin was somewhat unexpected.

CONCLUSIONS

It is evident from the above sections that we are presently of the opinion that the major contributions of the interactions between these aromatic or hydroxyaromatic compounds and the four substrates studied have been of the "donor-acceptor" type and that the contributions from hydrophobic bonding and hydrogen bonding were possibly of lesser magnitude. The general considerations discussed herein can, however, only be considered as speculative. Further work along these lines may bring to light additional factors which have not been evident in this limited study.

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Effects of Certain Ataraxic Agents on Mice Activity

By JAMES E. WEAVER† and TOM S. MIYA

A combination of a test for spontaneous motor activity (SMA) and rolling roller performance (RRP) was employed to evaluate certain centrally acting compounds on mice. These tests are useful for detecting compounds that selectively depress spontaneous activity without producing concomitant motor deficit.

CERTAIN drugs produce a selective depression of spontaneous motor activity (SMA) without concomitant neurological deficit. Kinnard and Carr (1), in this laboratory, proposed that tests on SMA conducted in the photocell activity cage and neurological deficit determination by rolling roller performance (RRP) might provide a rapid and useful means to differentiate between the depression produced by various central nervous system depressants. Using increasing dosage levels of drugs and employing a short (approximately fifteen minutes) waiting period, they observed severe depression of SMA with little effect on RRP for ectylurea (2-ethylcrotonylurea). A greater depression of RRP than SMA observed for meprobamate was attributed to the muscular relaxant properties of the drug.

The present study was designed to extend this work to determine whether the time interval employed was of sufficient duration to allow observation of drug effect.

EXPERIMENTAL

The circular photocell activity cages employed were those described by Kinnard and Carr (1) and the rolling roller was that described by Dunham and Miya (2). The method of training animals employed in the RRP tests and the activity ratios (treated/control) used for comparison in both tests were those used in the initial study (1).

Tests for the effect of drugs upon RRP were conducted by placing the animals which had received the drug by intraperitoneal injection on the rolling roller at zero, fifteen, thirty, forty-five, sixty, ninety, and one hundred and twenty minutes following drug administration. The time each animal remained on the roller was recorded in seconds up to one hundred and twenty seconds, after which time all remaining animals were removed. Animals to be tested were fasted for twelve hours prior to the test. They received water *ad libitum*, except during the testing procedure. Tests for the effect of the depressants on SMA were conducted by placing in a photocell activity cage a group of five drug-treated animals and in another similar cage a group of five control animals, and recording their activity during zero to fifteen, fifteen to thirty, thirty to forty-five, forty-five to sixty, sixty to ninety, and ninety to one hundred and twenty-minute intervals. The cross-over technique was utilized so that all control animals received the drug and all treated animals served as controls.

Male, albino Swiss mice weighing between 25–35 Gm. were used throughout the test. Aqueous solutions of chlorpromazine,[1] promazine,[1] and hydroxyzine,[2] and suspensions of ectylurea[3] and meprobamate[4] in 1% sodium carboxymethylcellulose (medium viscosity) were employed. The control animals received the respective vehicles and were studied simultaneously.

RESULTS

Chlorpromazine (Figs. 1 and 2) at both dosage levels (3 and 5 mg./Kg.) exhibited RRP depression as severe or more severe than the depression of SMA. SMA returned to control levels and during the ninety to one hundred and twenty-minute interval following 3.0 mg./Kg. it was supranormal. This was also observed to a marked degree with hydroxyzine.

Promazine was administered at dosage levels of 2.71 and 4.52 mg./Kg. (equal in phenothiazine base to 3.0 and 5.0 mg./Kg. of chlorpromazine). Both doses gave essentially identical results (see Fig. 3). RRP was depressed more than SMA in both instances and it is apparent that promazine has much

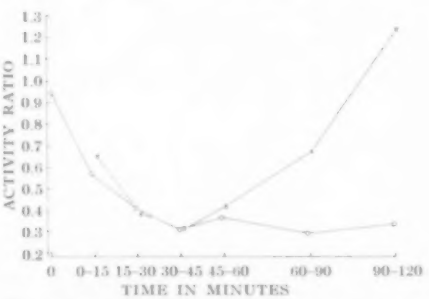

Fig. 1.—Effect of 3.0 mg./Kg. chlorpromazine on spontaneous motor activity (SMA) and rolling roller performance (RRP) on mice. x——x, SMA; o——o, RRP.

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[2] Generously supplied by J. B. Roerig & Co., New York, N. Y.
[3] Generously supplied by Miles-Ames Research Laboratories, Elkhart, Ind.
[4] Generously supplied by Wallace Laboratories, New Brunswick, N. J.

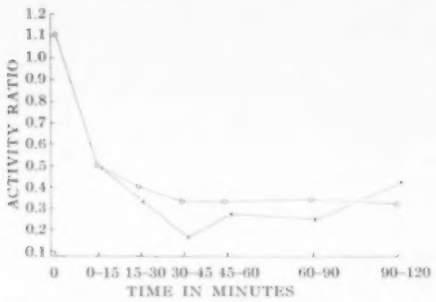

Fig. 2.—Effect of 5.0 mg./Kg. chlorpromazine on SMA and RRP on mice. x——x, SMA; o——o, RRP.

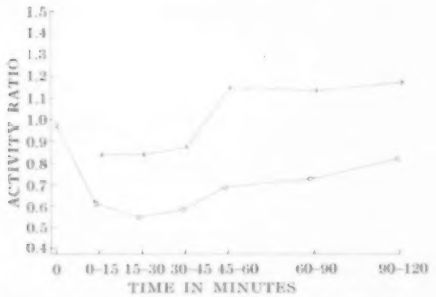

Fig. 3.—Effect of 4.52 mg./Kg. promazine on SMA and RRP on mice. 2.71 mg./Kg. gave essentially the same results. x——x, SMA; o—o, RRP.

less SMA-depressing qualities than does chlorpromazine.

Hydroxyzine, 32 mg./Kg., elicited a prompt depressant response of both SMA and RRP of approximately equal intensity. As can be observed in Fig. 4, drug action appears to wane in about forty-five minutes. However, the SMA increased markedly after this time. Whether this is due to drug action or initial suppression of orientation hyperactivity is not known.

Ectylurea, 50 mg./Kg., (Fig. 5) had no significant effect on RRP during the test period, but reduced SMA more than 50%; the effects lasted for the duration of the two-hour test period.

With meprobamate, 100 mg./Kg., a marked depression of SMA was observed (Fig. 6). The time of peak SMA depression corresponds closely to the time required for maximum concentration of radioactivity in rat brain tissue following intraperitoneal injection of carbon[14] carbamate labeled meprobamate (3). RRP was not appreciably affected by this dose.

DISCUSSION

Kinnard and Carr (1) observed that meprobamate produced a pattern similar to barbiturates, that is, reduction of both SMA and RRP. This was attributed, in part, to the skeletal muscle relaxant properties of meprobamate. The waiting period

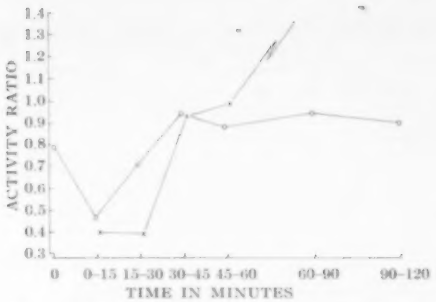

Fig. 4.—Effect of 32.0 mg./Kg. hydroxyzine on SMA and RRP on mice. x——x, SMA; o——o, RRP.

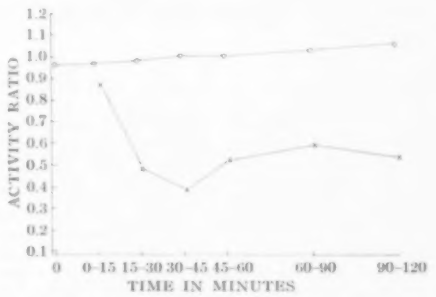

Fig. 5.—Effect of 50.0 mg./Kg. ectylurea on SMA and RRP on mice. x——x, SMA; o——o, RRP.

Fig. 6.—Effect of 100.0 mg./Kg. meprobamate on SMA and RRP on mice. x——x, SMA; o——o, RRP.

used in the original study was not sufficiently long to observe the deep depression of SMA produced by meprobamate. The inability to observe a distinct separation of effects upon SMA and RRP following meprobamate was apparently not due to the inherent property of the drug, but to the time interval employed in the original study. The shorter time interval employed allows quick determinations but may not allow for differences in lag time of various compounds.

According to this procedure of testing it is possible to detect compounds which have a differ-

ential effect on two areas of the brain; those which control spontaneous movements (awareness of environment) and those which control muscular coordination. This method appears to be particularly suited for screening drugs intended to be employed for daytime sedation.

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Relationship Between the Kinetics of the Acetylation and Excretion of Sulfathiazole or Other Sulfonamides and the Occurrence of Crystalluria

By EINO NELSON

The kinetics of the excretion and acetylation of sulfathiazole were studied in normal adult humans. Excretion of free sulfathiazole, its acetylation, and excretion of acetylated sulfathiazole were described by a kinetic model previously found to hold for the same processes in the disposition of sulfisoxazole and sulfamethylthiadiazole when 0.5-Gm. doses were ingested. When a 1.0-Gm. dose of sulfathiazole was ingested by one test subject, excretion of acetylated sulfathiazole could not be described in terms of the model. However, repetition of the test during the maintenance of a high water intake during the period of time when excretion rate of acetylsulfathiazole was at high values, gave results that could be interpreted in terms of the model. On the basis of equations developed to calculate maximum excretion rate of acetylsulfathiazole, it was apparent that crystalluria must have occurred during the first test with a 1.0-Gm. dose. The relationship between concentration of acetylated sulfonamide in urine, urine flow rate, and rate of excretion of acetylated sulfonamides was examined for the situation when sulfonamides are used in therapy. This examination allowed establishment of minimum urine flow rates during therapy with sulfathiazole, sulfisoxazole, and sulfamethylthiadiazole as a function of urine pH and dose level.

PREVIOUSLY MADE studies with sulfisoxazole (1) and sulfamethylthiadiazole (2) have shown that the disposition of these drugs by the human consists of two competitive first-order processes, one for acetylation and the other for excretion of free drug. The acetylated drug is eliminated in the urine by another first-order process that follows its acetylation. It was of interest to study another sulfonamide, sulfathiazole, to determine whether or not the same sequence of processes occurs in its disposition. The results of this study and some of their implications in the use of this drug and other sulfonamides in therapeutics are presented now.

EXPERIMENTAL

Conduction of Tests.—Adult humans in apparent normal health ingested 0.5- or 1.0-Gm. doses of finely powdered sulfathiazole in the morning on fasted stomachs. The drug was suspended in about 200 ml. of water prior to ingestion and a number of rinsings of the container sufficient to insure quantitative administration of the dose also ingested. No food was taken for at least one hour after ingestion. No special instructions in regard to water intake were given to test subjects ingesting 0.5-Gm. doses. Because of the results obtained in one test subject taking a 1.0-Gm. dose of sulfathiazole, the test was repeated and a water intake of 500 ml./hr. maintained for the first eight hours of the experiment.

Urine was collected at two, four, six, eight, ten, twelve, twenty-four, and thirty-six hours after ingestion of the doses. The volume of the collections were noted and aliquots taken and stored at about 2° until assay by the Bratton and Marshal procedure for free and total sulfonamide in the urine.[1]

The designation, age in years, weight in Kg and sex of the subjects participating in the tests were, respectively: E-41-77-M; C-29-57-F; P-30-72-M; and B-32-66-M. All subjects ingested 0.5-Gm. doses of drug in a single test. On two separate occasions, subject E ingested 1.0-Gm. doses of drug. On one of these occasions the 500 ml./hr. water intake during the first eight hours of the test was maintained as previously discussed.

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[1] The author wishes to acknowledge the most efficient assistance of Mr. Gerald Reichelderfer in the conductance of these assays.

TABLE I.—CUMULATIVE MG. FREE (F) AND ACETYLATED (A) SULFATHIAZOLE AND ML. URINE EXCRETED BY TEST SUBJECTS IN VARIOUS TIMES IN HOURS[a]

	Mg. Excreted															
	2.0		4.0		6.0		8.0		10.0		12.0		24.0		36.0	
Subject	F	A	F	A	F	A	F	A	F	A	F	A	F	A	F	A
E[b]	98	13	277	57	421	92	527	115	600	140	649	152	755	207	800	232
E	56	3	154	14	225	24	265	33	306	40	331	47	387	64	408	67
E[b]	72	8	231	27	375	54	474	83	549	106	632	125	755	190	786	213
C	54	2	163	6	252	26	313	36	350	48	375	58	438	78	451	79
P	52	3	160	15	259	30	312	46	348	54	376	60	444	78	452	79
B	55	7	145	28	210	52	252	66	282	81	308	95	362	132	371	141

	Ml. Urine						
Subject	2.0	4.0	6.0	8.0	10.0	24.0	12.0	36.0
E[b]	170	350	600	800	1,350	2,520	3,000	4,640
E	100	270	455	595	785	2,010	3,940	6,155
E[b]	1,540	2,640	3,680	4,600	4,740	4,920	6,030	7,440
C	210	310	380	580	740	790	1,230	2,410
P	50	200	350	850	1,190	1,290	1,730	3,750
B	60	210	340	700	830	1,090	2,730	3,240

[a] Acetylated drug given in terms of free drug. [b] 1.0-Gm. dose. All other data from 0.5-Gm. doses.

Results.—The amounts of free and acetylated sulfathiazole and ml. urine excreted in various times by the test subjects are given in Table I.

DISCUSSION

Mathematical Analysis.—In the kinetic model previously found to account for the disposition of sulfisoxazole (1) and sulfamethylthiadiazole (2), excretion of acetylated drug, after a correction for amount excreted during the period when drug was being absorbed and attaining equilibrium in the body, was given by the following expression.

$$Sae = A(1 - \exp[-Kt']) - B(1 - \exp[-k_2t']) \quad \text{(Eq. 1)}$$

where the terms have the following meanings:

Sae = amount of acetylated sulfonamide in mg. free sulfonamide excreted after the time taken as zero time

$A = \dfrac{k_1k_2S^\circ}{K(k_2 - K)}$

$B = \dfrac{k_1S^\circ}{(k_2 - K)} - Sa^\circ$

K = the rate constant in reciprocal hours for elimination of drug from the body, i. e., the sum of rate constants for elimination of free drug and for acetylation ($K = k_1 + k_2$)

k_1 = the rate constant in reciprocal hours for acetylation

k_2 = the rate constant in reciprocal hours for elimination of acetylated drug

k_3 = the rate constant in reciprocal hours for excretion of free drug

S° = the amount of unchanged drug in mg. in the body at the time taken as zero time

Sa° = the amount of acetylated drug in mg. free drug in the body at the time taken as zero time

t' = time in hours after zero time and in the present experiment, $t' = t - 2$ where t is clock time.

Excretion of unchanged drug was given by Eq. 2

$$Se = S^\circ f(1 - \exp[-Kt']) \quad \text{(Eq. 2)}$$

In Eq. 2, Se was the mg. unchanged drug excreted after zero time and f the fraction of a dose reaching circulation that was excreted unchanged in a time infinite in terms of the experiment.

Excretion data from the experiments were fitted to Eqs. 1 and 2 by the procedures described in the report of sulfisoxazole (1). Excellent agreement between theoretically calculated excretion by these expressions and excretion experimentally observed was obtained when data from 0.5-Gm. doses was examined. This agreement is shown in Fig. 1 for subject C. The agreement was similar from data obtained for subjects E, P, and C, and in past work with other sulfonamides (1, 2). The values of the constants in Eqs. 1 and 2 for each of the test subjects are listed in Table II. The data point times of t' = four, six, eight, and ten hours were used to find the mean value of k_2 (1).

In one of the two tests in which subject E took a 1.0-Gm. dose of drug and only normal amounts of water were ingested during the day, excretion of unchanged drug was well described by Eq. 2. When experimental data on excretion of acetylated drug were examined (1) in an attempt to find the rate constant for excretion of this material, no value could be found that would result in even reasonable agreement between calculated and experimentally

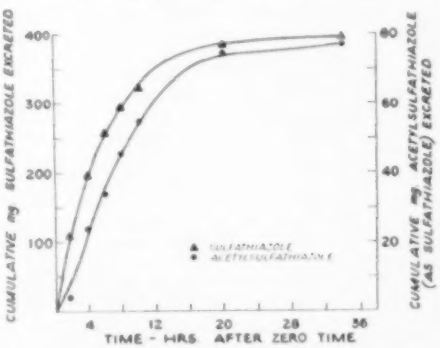

Fig. 1.—Agreement between theoretical and experimentally observed excretion of sulfathiazole and acetylsulfathiazole following a single 0.5-Gm. dose; subject C. Solid lines are theoretical excretion values and plotted points are experimental values.

TABLE II.—RATE AND OTHER CONSTANTS DETERMINING ACETYLATION AND EXCRETION OF SULFATHIAZOLE BY TEST SUBJECTS

Constant	E[a]	E[a,c]	E	C	P	B	Means
k_1, hr.$^{-1}$	0.035	0.029	0.021	0.026	0.026	0.044	0.030
k_2, hr.$^{-1}$	[b]	0.326	0.408	0.356	0.519	0.351	0.392
k_3, hr.$^{-1}$	0.120	0.106	0.131	0.146	0.146	0.117	0.128
K, hr.$^{-1}$	0.115	0.135	0.152	0.176	0.172	0.161	0.158
S^0, mg.	905	908	410	466	469	435	...
S_T,[c] mg.	15	11	7	7	7	15	...
f	0.776	0.786	0.859	0.850	0.850	0.726	0.808

[a] 1.0 Gm. dose; all other data from 0.5-Gm. doses. [b] Excretion of acetylated drug was not by a first-order process. [c] Test with at least 500 ml. water per hour intake during first eight hours of experiment.

observed amounts of drug excreted. The procedure for finding the value of k_2 consists of equating experimental values of excreted acetylated drug to Eq. 1 and, after evaluation of other constants, finding a value of k_2 that satisfies this value. This is done at several data point times and the values obtained averaged. The procedure resulted in values of k_2 which continued to decrease from the data point time (t') of two hours to twenty-two hours. The cumulative amount of acetylated drug excreted *vs.* time plot was examined for indications of linearity which would indicate a zero-order process in either acetylation or excretion of acetylated drug. There was no clear indication of this occurrence. The fact that Eq. 2 described excretion of free drug rules out the possibility that a zero-order process was involved in acetylation, since such a process would be competitive with the first-order process by which excretion of unchanged drug occurred and hence prevent description of this excretion by Eq. 2. Biological conjugations have been observed to proceed by zero-order processes when only a limited supply of the conjugating substance is present (3). The lack of linearity in the cumulative excretion curve of acetylated drug also ruled out a zero-order process in excretion that would indicate a saturation of kidney function. Excretion of acetylated drug would not be described by a first-order process if the acetylation process were reversible. This could not have been the case because again excretion of unchanged drug would not have been described by Eq. 2. The nonconformance of acetylsulfathiazole excretion to the model postulated left open one relatively simple possibility. This was that acetylsulfathiazole precipitated in the kidney due to a low urine flow rate. This occurrence is common in the use of sulfathiazole and may lead to hematuria which was not observed in gross observations of the specimens obtained in the present study. Before attempting the fit of acetylsulfathiazole excretion to more complex models, some preliminary calculations were made and these indicated that the volume of urine passed during the time the maximum amount of acetylsulfathiazole was in the body was less than that necessary to keep acetylsulfathiazole in solution at usual urine pH's. This led to repeating the test with a 1.0-Gm. dose of sulfathiazole and high level of water intake.

Maximum Excretion Rate of Acetylsulfathiazole From Single-Dose Administration and Relationship to Urine Concentration.—The concentration of acetylsulfathiazole in urine may be calculated at any time by the following

$$Cu = \frac{d.Sae/dt}{dU/dt} \qquad \text{(Eq. 3)}$$

where Cu is the urine concentration, $d.Sae/dt$ is the excretion rate of acetylsulfathiazole, i.e., the derivative of Eq. 1

$$d.Sae/dt = AK \exp[-Kt'] - Bk_2 \exp[-k_2t'] \qquad \text{(Eq. 4)}$$

and dU/dt is the urine flow rate. Cu may be taken in mg./ml., acetylsulfathiazole excretion rate in mg./hr., and urine flow rate in ml./hr. Knowing the solubility of acetylsulfathiazole, this solubility can be compared to that calculated by Eq. 3, and whenever the latter value exceeds the former, precipitation in the kidney may be expected. The value of Cu from Eq. 3 is of interest when acetylsulfathiazole excretion rate has its maximum value. The time of occurrence of this maximum rate may be found equating the derivative of Eq. 4 to zero and solving for t'. Hence

$$t'_{max} = \frac{\log_e[Bk_2^2/AK^2]}{k_2 - K} \qquad \text{(Eq. 5)}$$

Equation 5 may be substituted in 4 to obtain after rearrangement

$$R_{max} = Bk_2 \left(\frac{k_2 - K}{K}\right) \left(\frac{AK^2}{Bk_2^2}\right)^{\frac{k_2}{k_2 - K}} \qquad \text{(Eq. 6)}$$

where R_{max} is the maximum excretion rate of acetylsulfathiazole.

In the test where subject E ingested a 1-Gm. dose of sulfathiazole and maintained a water intake of 500 ml./hr., a value of the rate constant k_2 was obtained from the excretion data that allowed description of acetylsulfathiazole excretion by Eq. 1. The fit of experimental values of free and acetylated sulfathiazole excretion to the values predicted by Eqs. 2 and 1, respectively, is shown on Fig. 2. Using the various parameters describing excretion of acetylated drug as listed in Table II for this test, the maximum excretion rate of acetylated drug occurred, by Eq. 5, 4.2 hours after zero time (clock time = 6.2 hours). By Eq. 6, the maximum rate of excretion was 15 mg./hr. This rate would require, by Eq. 3, a urine flow rate of about 200 ml./hr. if the solubility of acetylated sulfathiazole is calculated at the average urine pH of 5.85 (3) by the method of Krebs and Speakman (4). Thus the urine output of about 500 ml./hr. that was maintained during the appropriate time of this test (Table I) was more than sufficient to prevent crystalluria.

In the first test with the 1-Gm. dose of sulfathiazole, subject E's average urine flow rate was only 100 ml./hr. between six and eight hours after the start of the test. It was reasonable to expect

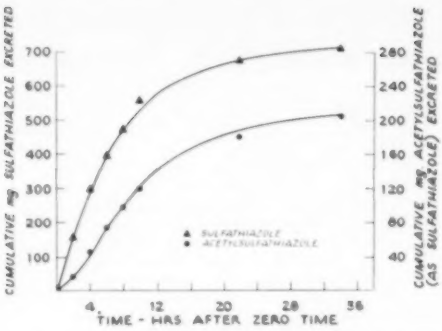

Fig. 2.—Agreement between theoretical and experimentally observed excretion of sulfathiazole and acetylsulfathiazole following a single 1.0-Gm. dose; water intake 500 ml./hr. during first eight hours of test; subject E. Solid lines are theoretical excretion values and plotted points are experimental values.

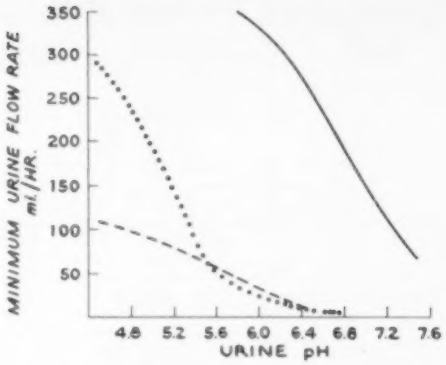

Fig. 3.—Minimum urine flow rates needed to be maintained in order to prevent precipitation of acetylsulfonamides in urine plotted as a function of urine pH. Solid line, acetylsulfathiazole, $k_1 = 0.027$ hr.$^{-1}$; dotted line, acetylsulfisoxazole, $k_1 = 0.023$ hr.$^{-1}$; dashed line, acetylsulfamethylthiadiazole, $k_1 = 0.045$ hr.$^{-1}$. Curves computed assuming a constant level of 1 Gm. unchanged drug in the body.

that crystalluria occurred and that this was the reason no first-order rate constant for excretion of acetylsulfathiazole could be found. The maximum excretion rate of acetylsulfathiazole in the first test was probably higher than the 15 mg./hr. calculated above for the second test because the rate constant for acetylation (k_1) was greater. Furthermore, the value of k_2 for excretion was substantially higher from calculations at the first two data point times in the first test.

No hematuria was observed in casual gross examination of urine specimens of subject E obtained during the first test with a 1-Gm. dose. However, it should not be unusual to expect that this result of crystalluria occurs only when precipitation is massive.

Excretion Rate of Acetylated Sulfonamides with Therapeutic Dosage Regimens.—It is usual in therapy with sulfonamides to give an initial loading dose which is followed by dosage with smaller amounts at regular time intervals which keep the body level of sulfonamide at an approximately constant value. At constant body level of sulfonamide, the level of acetylated drug will also become fixed at a constant level, meaning that acetylated drug will eventually be excreted at a constant rate. It was shown in a preliminary communication concerning the present work (5) that under these conditions of administration the relationship between urine flow rate, rate constant for acetylation, body level of drug, and urine concentration was given by Eq. 7 which follows

$$dU/dt = k_1 g/Cu \qquad \text{(Eq. 7)}$$

In Eq. 7, g is the body level of drug. The relationship between minimum urine flow rate and urine pH is shown in Fig. 3 for several sulfonamides. Data for calculation were available from the present and previous work in this laboratory (1, 2, 5). In the plot of Fig. 3, it was assumed that a constant amount of 1 Gm. of drug was in the body. Doubling this amount would mean that the minimum urine flow rate at a given urine pH would have to be doubled.

Other Products of Sulfathiazole's Metabolism.—According to Williams (6) hydroxylation of the benzene ring occurs to a very minor extent and the resulting product is probably 3-hydroxysulfathiazole. Since the extent of this reaction is minor, no significant error is introduced into the results of the present work by not accounting for its production in the mathematical description of sulfathiazole's metabolism.

SUMMARY

Studies were conducted to determine the value of various rate constants and other parameters controlling the rate of acetylation and excretion of sulfathiazole. An expression was developed to calculate maximum excretion rate of acetylated sulfathiazole and other acetylated sulfonamides and used to predict the potentiality of crystalluria following single doses of drug. An expression giving maximum value of excretion rate of acetylated sulfonamides during therapy with these materials was used to calculate minimum urine flow requirements for prevention of crystalluria.

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Physical Chemical Study of the Distribution of Some Amine Salts Between Immiscible Solvents

By GAUTAM J. DIVATIA and JOHN A. BILES

Several alkyl amine salts of tropaeolin 00 have been isolated. The apparent distribution constant, *K*, of these salts was found to be governed by the molecular weight and branching of the aliphatic amine, the relative concentrations of the amine and dye, and the dielectric constant of the solvent. Equilibria and absorbance studies showed that the salts existed as monomeric ion-ion pairs in the nonpolar phase. Quantitative treatment of the *K* values revealed a direct relationship between the log *K* value and the molecular weight of the amine. The apparent distribution constants are indicative of the complexity which a pharmaceutical amine should possess before a colorimetric method of analysis can be developed by extracting the dye salt with a suitable immiscible solvent. The true distribution constant, K_d, was determined for di-iso-amylamine tropaeolinate.

MANY IMPORTANT pharmaceuticals which have been introduced are amine derivatives. These compounds include the sympathomimetic amines, the antihistamines, many of the tranquilizers, the quaternary ammonium compounds, and other nitrogenous bases. Simple assay procedures are not available for many of these compounds. Colorimetric methods of analysis for ionic organic compounds by formation of salts of these organic compounds with suitable dyes have been reported (1–6). These colorimetric methods of analysis depend on the formation of a salt with the ionic agent and a dye which is of opposite ionic type.

After forming the salt of the dye in aqueous solution, a suitable nonpolar solvent is chosen so that this salt is extracted quantitatively into the nonaqueous solvent. Mukerjee (2) discussed the factors leading to maximum dye salt formation and its extraction by the solvent. Helgren, Theivagt, and Campbell (4) applied Mukerjee's work in the development of an assay for hexocyclium methylsulfate. Patel and Miller (3) stated that maximum salt formation would take place when the pH of the system was maintained at least two units above and below the pKa and pKb values of the organic substance and the dye used for salt formation.

It has been assumed that extraction of the salt of the dye from the aqueous system into the nonpolar system is good if the molecular weight of the solute is high. In the development of the suitable assay emphasis has been placed on complete extraction with a reasonable quantity of immiscible solvent. Studies concerning the relative solubility and factors controlling this have been lacking. The purpose of this communication is to report the results of equilibria studies of some amine tropaeolinates between immiscible solvents. The relationship of the molecular weight and structure of amines and their effect on the apparent distribution constants of the salts are reported. The determination of the true distribution constant for one salt is described.

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Presented to the Scientific Section, A.Ph.A., Cincinnati meeting, August 1959.

EXPERIMENTAL AND DISCUSSION

Reagents.—The aliphatic amines were obtained from Eastman Kodak, Matheson, Coleman and Bell, and the K. and K. Laboratories. The amines used, their respective refractive indexes, boiling points, molecular weights, and experimental neutral equivalents are reported in Table I.

The methylene chloride, chloroform, and carbon tetrachloride were purchased from Baker and Adamson and were reagent grade. Methylene blue and tropaeolin 00 were purchased from National Aniline Division, Allied Chemical and Dye Corp. The tropaeolin 00 was recrystallized from water four times.

Solvents and Solutions.—Four nonpolar solvents were used in the extraction studies. Each of these solvents was saturated with distilled water before use. Two of the solvents included methylene chloride and chloroform. Two additional solvents were prepared by mixing chloroform and carbon tetrachloride. One blended solvent contained equal parts by volume of chloroform and carbon tetrachloride; the other blended solvent contained two volumes of chloroform and one volume of carbon tetrachloride.

The water which was used in diluting the stock solutions of the different amines and in the preparation of the dye solution was distilled and then saturated with one of the four nonpolar solvents, the choice depending on which extracting solvent was used.

The aqueous solution of the tropaeolin 00 was prepared by dissolving 0.1501 Gm. of the dye, previously dried to constant weight, in enough of water saturated with the extracting solvent to

TABLE I.—SOME PHYSICAL CONSTANTS OF SOME ALIPHATIC AMINES

Amine	Refractive Index[a]	Boiling Point, °C.[b]	Molecular Weight	Neutral Equivalent[c]
Di-*n*-propylamine	1.4035	108–110	101.18	99.65
Di-iso-propylamine	1.3916	82.5	101.18	94.3[d]
Di-*n*-butylamine	1.4174	157–158	129.23	131.14
Di-iso-butylamine	1.4080	137–139	129.23	125.6
Di-*n*-amylamine	1.4260	204	157.28	156.35
Di-iso-amylamine	1.4227	186–189	157.28	157.6
Di-*n*-hexylamine	1.4340	234–237	185.33	187.15

[a] The refractive indexes were determined with an Abbe refractometer at 25°, using white light. [b] The temperature of the boiling points are uncorrected. [c] Methyl red was used as the indicator. [d] Distillation was not a successful method to further purify the di-iso-propylamine.

make 2 L. of solution. The pH of this solution was 6.1 ± 0.05.

A 0.1*N* sodium acetate-acetic acid buffer solution was made and used to prepare stock solutions of the amines. The pH of the buffer was 4.75. The solutions of amines were prepared by placing 1 mmole of the amine, accurately weighed, into a 100-ml. volumetric flask. Five milliliters of ethanol was added to solubilize the amine. A 25-ml. amount of the buffer and sufficient distilled water were added to make 100 ml. This stock solution was then used to prepare a solution containing either 1 or 2 μm. of the amine per 5 ml. of solution. This latter solution was made to volume with distilled water and saturated with the extracting solvent used in determining the apparent distribution constants. The pH of this diluted amine solution was 5.0 ± 0.05.

A 3% sodium borate solution was prepared by dissolving 60 Gm. of sodium borate, U.S.P., in enough distilled water to make 2 L. of solution. The solution was filtered before using.

Apparatus.—All volumes of solutions were measured using TD pipets or burets. The absorbance of the solutions was measured using a Spectronic 20 colorimeter. The melting points of the isolated salts were determined with a Kofler melting point block. The dielectric constant of the nonpolar liquids was determined with a Heathkit direct reading capacity meter using an aluminum condenser. The pH of the aqueous solutions was determined using a Beckman pH meter.

Procedure.—The maximum absorbance of the tropaeolin 00 in aqueous solution was found to be 440 mμ. This maximum was not shifted when aliphatic amines were added to the tropaeolin 00 solution or when the solution contained up to 2.5% sodium borate. Beer's law was obeyed for the aqueous solutions for concentrations varying from an absorbance of 0.05 to 0.60. It was found that 1.0 μm. of tropaeolin 00 in 100 ml. of aqueous solution showed an absorbance of 0.27.

The apparent and true partition constants, K and K_d, were determined in the following manner: an equal number of μm., usually 2, of the amine and tropaeolin 00 was added to a glass-stoppered Erlenmeyer flask. However, when partitioning was small, up to 8 μm. of amine and 4 μm. of tropaeolin 00 were used. The aqueous solution in the Erlenmeyer flask was brought to 40 ml. by adding water saturated with the appropriate nonpolar solvent. To the aqueous solution was added 40 ml. of the appropriate nonpolar solvent which had previously been saturated with distilled water. The flask was stoppered and shaken for 10 minutes to effect equilibrium. The shaking time was determined as outlined by Helgren (4). After shaking in the Erlenmeyer flasks, the liquids were transferred to glass-stoppered separators. The nonpolar and aqueous phases were separated.

The aqueous phase was then diluted with a 3% sodium borate solution by bringing a specific volume of the aqueous phase to the total volume in the chosen volumetric flask. The absorbance of this solution was measured at 440 mμ. The amine tropaeolinate in the nonpolar phase was treated with the sodium borate solution to liberate the tropaeolin 00 from the nonpolar phase. This was done by placing an aliquot of the nonpolar solution, usually 20 ml., into a separator and extracting with two volumes of the sodium borate solution. The aqueous extract was collected in a suitable volumetric flask and brought to volume. The absorbance of this solution was measured. The μm. of the tropaeolin 00 in each of the solutions were calculated from the absorbance values. The micromolar concentration of amine tropaeolinate extracted by the nonpolar solvent was referred to as c_2 or c_o. The amine tropaeolinate was also referred to as BHTr. The micromolar concentration of tropaeolin 00 remaining in the aqueous solution after partitioning was referred to as c_2 or c_w. In the aqueous phase the tropaeolin 00 was also referred to as Tr^-. In almost all the experiments it was found that the sum of c_2 and c_3 was slightly less than the original number of micromoles of tropaeolin 00 added. This was explained by the loss in the transferring and the extracting procedures. The micromolar concentration of amine remaining in the aqueous solution after partitioning was referred to as c_1. It was also referred to as BH^+. Since the amine tropaeolinate formed from a mono acid and mono base, it was concluded that the concentration of the amine in the nonpolar phase was equivalent to the tropaeolin 00 in the nonpolar phase. The difference between the total concentration of amine added and c_3 represented the concentration of c_1.

The amine in aqueous solution, pH 5.0, was determined to be insoluble in the nonpolar phase using the following procedure. A specific micromolar concentration of the amine solution was placed in enough distilled water to make a volume of 20 ml. An equal volume of the nonpolar solvent was added. The heterogeneous solution was shaken for 10 minutes and the nonpolar phase removed. The presence of any amine in the nonpolar phase was tested by adding 20 ml. of water containing 1 μm. of tropaeolin 00. The mixture was shaken for 10 minutes. Any amine present in the nonpolar phase would partition between the water and the nonpolar

solvent. In the aqueous phase amine tropaeolinate would be formed and then partition. If this occurred the nonpolar phase would become an orange-yellow color showing a maximum absorbance of 405 mμ. In all cases it was observed that the nonpolar layer was colorless. The sodium salt of the dye was found to be insoluble in the four nonpolar solvents used.

Apparent and True Partition Coefficients.—Nernst emphasized that the distribution law was applicable only to the molecular species common to both phases (7). In order to calculate the true partition or distribution coefficient, the solute must exist as a simple monomer in the immiscible solvents when referring to the chemical equation given below. Calculation of the true partition or distribution coefficient becomes complex if there is association or ionization of the solute in either or both of the solvents (8–10). The apparent distribution coefficient is determined when partitioning is established between species not common to the immiscible phases.

$$\underset{c_1}{BH^+_{(aq.)}} + \underset{c_2}{Tr^-_{(aq.)}} \overset{K_f}{\underset{K_i}{\rightleftarrows}} \underset{c_3}{BHTr_{(aq.)}} \overset{K_d}{\rightleftarrows} BHTr_{(nonpolar\ solvent)} \quad \text{(Chem. Eq.)}$$

To be able to calculate the true partition coefficient from the distribution studies of the amine tropaeolinate (BHTr), the formation constant, K_f, or the ionization constant, K_i, of BHTr in the aqueous phase would have to be determined. From this the true partition coefficient, K_d, could be calculated. However, should ionization be complete in the aqueous phase, the apparent partition coefficient would be determined since the solute species would be different in the immiscible phases.

Determination of the Apparent Partition Coefficient.—According to the chemical equation, the apparent partition coefficient would be governed by the ionic species in the aqueous phase (i.e., c_1 and c_2) and the BHTr (i.e., c_3) in the nonpolar phase. The ion species can be guaranteed by adjusting the pH of the aqueous solution. The solution was adjusted to a pH of 4.95–5.0. The pKb values for the aliphatic amines are of the order of the ammonium hydroxide value. The pKa value of tropaeolin 00 is 2.10. At a pH of 5.0 the amount of dissociation of the amine and tropaeolin 00 can be calculated by substituting the above values into the Henderson equation. Calculations indicated that dissociation of the amines and tropaeolin 00 was more than 99.5%.

If the BHTr in the aqueous phase is completely dissociated into BH^+ and Tr^-, but exists as a monomer in the nonpolar phase, the sum of the chemical potential of the ions in the aqueous layer is equal to the chemical potential of the undissociated solute in the nonpolar layer. In the nonpolar solvent

$$\mu_s = \mu^0_s + RT \ln c_s\gamma_s \qquad \text{(Eq. 1)}$$

and in the aqueous layer

$$\mu_w = \mu^0_{BH^+} + RT \ln C_{BH^+}\gamma_{BH^+} + \mu^0_{Tr^-} + RT \ln C_{Tr^-}\gamma_{Tr^-} \qquad \text{(Eq. 2)}$$

At equilibrium

$$\mu_s = \mu_w \qquad \text{(Eq. 3)}$$

and

$$\mu^0_s + RT \ln c_s\gamma_s = \mu^0_{BH^+} + RT \ln C_{BH^+}\gamma_{BH^+} + \mu^0_{Tr^-} + RT \ln C_{Tr^-}\gamma_{Tr^-} \qquad \text{(Eq. 4)}$$

or

$$\frac{c_s\gamma_s}{(C_{BH^+}\gamma_{BH^+})(C_{Tr^-}\gamma_{Tr^-})} = e^{-\frac{(\mu^0_w - \mu^0_{BH^+} - \mu^0_{Tr^-})}{RT}} = K \qquad \text{(Eq. 5)}$$

If the more concentrated solution is dilute then the activity coefficient, γ, approaches unity and

$$K = \frac{c_s}{(C_{BH^+})(C_{Tr^-})} \qquad \text{(Eq. 6)}$$

If

$$C_{BH^+} = C_{Tr^-} \qquad \text{(Eq. 7)}$$

then

$$K = \frac{c_s}{C^2_w} \qquad \text{(Eq. 8)}$$

or

$$K' = \frac{\sqrt{c_s}}{c_w} \qquad \text{(Eq. 9)}$$

Having previously defined

$$c_s = c_3 \qquad \text{(Eq. 10)}$$

$$C_{BH^+} = c_1 \qquad \text{(Eq. 11)}$$

$$C_{Tr^-} = c_2 \qquad \text{(Eq. 12)}$$

then

$$K = \frac{c_3}{c_1c_2} \qquad \text{(Eq. 13)}$$

By definition, K in Eq. 13 will be the apparent partition coefficient. In the manner described for the procedure for determining the apparent partition coefficient, the data were obtained using the symmetrical straight and branched chain secondary amines. The values are recorded in Tables II and III. The concentrations recorded in columns 4, 5, and 6 represent concentrations in μm. per 40 ml. These concentrations were corrected to moles per liter and these values were used to calculate the K values, using Eq. 13. Ferguson's work was then extended to this study to establish a relationship between the apparent distribution constant of the amine tropaeolinates and their respective molecular weights (11). The log K values were plotted against the molecular weight of the amine tropaeolinates. The plots are shown in Figs. 1 and 2.

It was anticipated that the di-iso-amine tropaeolinates would be relatively more soluble in the aqueous layer than the di-*n*-amine tropaeolinates, since a greater degree of branching favors greater water solubility (12). Comparison of the data in Tables II and III verified the anticipation.

Equation 13 was further tested with an extensive study of the distribution of di-iso-amylamine tropaeolinate. The purpose of this was to determine if the BHTr(i.e., c_3) as written in the chemical equation existed as the monomer. In addition, it was necessary to show that the K value was governed by the ion species (i.e., c_1 and c_2) in the aqueus phase. The concentration of one of the species in the aqueous phase was held constant, the concentra-

TABLE II.—DISTRIBUTION OF AMINE TROPAEOLINATES BETWEEN NONPOLAR SOLVENTS AND WATER

Amine	Amine,[a] μm.	Tropaeolin 00[a], μm.	c_3	c_2	c_1	K[b]
			Methylene Chloride			
Di-*n*-propylamine	4.0	4.0	0.311	3.50	3.69	0.965
Di-*n*-butylamine	2.0	2.0	0.600	1.30	1.40	13.20
Di-*n*-amylamine	1.0	1.0	0.675	0.339	0.325	244.5
			Chloroform			
Di-*n*-propylamine	8.0	4.0	0.333	3.74	7.67	0.464
Di-*n*-butylamine	2.0	2.0	0.448	1.55	1.55	7.48
Di-*n*-amylamine	1.0	1.0	0.548	0.400	0.452	121.5
			$CHCl_3$:CCl_4—2:1			
Di-*n*-propylamine	8.0	4.0	0.077	3.98	7.92	0.098
Di-*n*-butylamine	2.0	2.0	0.114	1.87	1.89	1.29
Di-*n*-amylamine	1.0	1.0	0.290	0.667	0.710	24.5
Di-*n*-hexylamine	1.0	1.0	0.695	0.261	0.305	348.0
			$CHCl_3$:CCl_4—1:1			
Di-*n*-butylamine	4.0	4.0	0.254	3.74	3.75	0.723
Di-*n*-amylamine	2.0	2.0	0.584	1.36	1.43	12.06
Di-*n*-hexylamine	1.0	1.0	0.597	0.348	0.403	169.8

[a] Micromolar concentration per 40 ml. of aqueous solution. [b] K was calculated by correcting all concentrations to moles per liter.

TABLE III.—DISTRIBUTION OF AMINE TROPAEOLINATES BETWEEN NONPOLAR SOLVENTS AND WATER

Amine	Amine,[a] μm.	Tropaeolin 00,[a] μm.	c_3	c_2	c_1	K[b]
			Methylene Chloride			
Di-iso-propylamine	4.0	4.0	0.197	3.73	3.80	0.552
Di-iso-butylamine	2.0	2.0	0.462	1.59	1.54	8.02
Di-iso-amylamine	1.4	1.0	0.638	0.348	0.762	102.8
			Chloroform			
Di-iso-propylamine	8.0	4.0	0.193	3.85	7.81	0.258
Di-iso-butylamine	2.0	2.0	0.297	1.73	1.70	4.03
Di-iso-amylamine	1.0	1.0	0.463	0.500	0.537	68.9
			$CHCl_3$:CCl_4—2:1			
Di-iso-butylamine	4.0	4.0	0.246	3.73	3.75	0.703
Di-iso-amylamine	2.0	2.0	0.653	1.30	1.35	14.92
			$CHCl_3$:CCl_4—1:1			
Di-iso-butylamine	4.0	4.0	0.116	3.70	3.70	0.339
Di-iso-amylamine	2.0	2.0	0.389	1.59	1.61	6.07

[a] Micromolar concentrations per 40 ml. of aqueous solution. [b] K was calculated by correcting all concentrations to mole per liter.

TABLE IV.—EFFECT OF VARYING CONCENTRATIONS OF REACTANTS ON THE EXTRACTION OF DI-ISO-AMYLAMINE TROPAEOLINATE BY METHYLENE CHLORIDE[a]

Amine Concn.	Tropaeolin 00 Concn.	c_3	c_2	c_1	c_1c_2	k'[b]
0.6	1.0	0.356	0.618	0.244	0.151	2.39
0.8	1.0	0.434	0.548	0.366	0.201	2.16
1.2	1.0	0.562	0.403	0.638	0.258	2.18
1.4	1.0	0.638	0.348	0.762	0.265	2.41
1.0	0.6	0.335	0.229	0.665	0.153	2.20
1.0	0.8	0.438	0.321	0.562	0.181	2.43
1.0	1.0	0.517	0.448	0.497	0.222	2.33

[a] Concentrations are expressed as μm. per 40 ml. [b] The value k' was determined by dividing c_3 by c_1c_2.

tion of the second species in the aqueous phase was varied, and the concentrations of c_1, c_2, ane c_3 were determined after partitioning was complete. The data obtained from the results of the variation of concentrations of c_1 and c_2 are recorded in Table IV, and the plot of the data is shown in Fig. 3. Examination of the plot in Fig. 3 indicates that the concentration c_3, plotted against the product c_1c_2 is a constant, and is a test of Eq. 13. The slope is termed $1/k'$. This constant was determined by using the micromolar concentrations in 40 ml. If the concentrations were corrected to moles per liter, then the slope would be equvalent to the reciprocal of the apparent distribution constant.

The data in Table IV and the regression line in Fig. 3 indicate that BHTr in the nonpolar phase exists as the ion-ion paired monomer. If this is true, then the pH of the aqueous phase should not change significantly after partitioning has occurred, and the dielectric constant of the nonpolar phase should not change significantly after BHTr has dissolved. The pH of the aqueous residues was determined as well as the dielectric constant of the nonpolar solvent and the nonpolar solution. The determined data are recorded in Table V. It is noted in Table V that

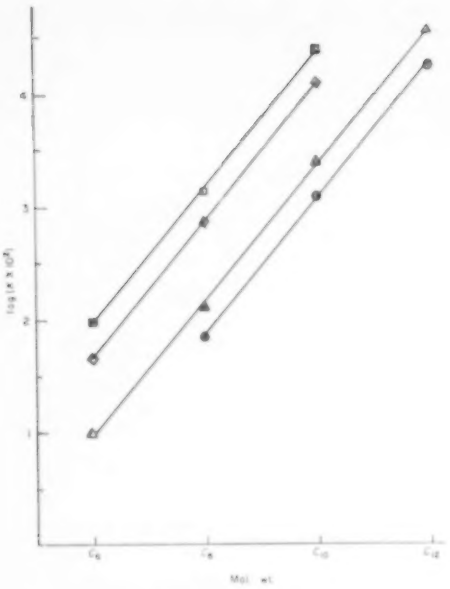

Fig. 1.—The apparent distribution constants of di-*n*-amine tropaeolinates between methylene chloride (□——□), chloroform (◇——◇), $CHCl_3$:CCl_4—2:1 (△——△), $CHCl_3$:CCl_4—1:1 (○——○), and water as related to molecular weight.

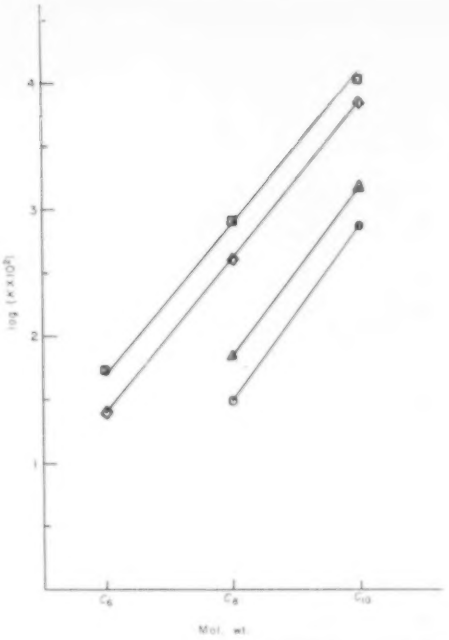

Fig. 2.—The apparent distribution constants of di-iso-amine tropaeolinates between methylene chloride (□——□), chloroform (◇——◇), $CHCl_3$:CCl_4—2:1 (△——△), $CHCl_3$:CCl_4—1:1 (○——○), and water as related to molecular weight.

different aliphatic amines were used. In addition, methylene blue was used. This dye was chosen because it is an amine and is insoluble in chlofororm. As previously stated, tropaeolin 00 is insoluble in chloroform. The methylene blue and tropaeolin 00 were mixed in aqueous solution, pH 5.0, and extracted with chloroform. After partitioning both immiscible solutions were green. The aqueous layer absorbed at maxima of 660 mμ (characteristic of methylene blue) and 440 mμ (characteristic of tropaeolin 00). The chloroformic solution absorbed at maxima of 650 and 405 mμ. A chloroformic solution of di-iso-amylamine tropaeolinate absorbed at a maximum of 405 mμ. These mμ values in the immiscible phases indicate that an ion-ion pair in the nonpolar phase is quite plausible. The dielectric constants of the nonpolar solvent and nonpolar solution support the consideration of the ion-ion pair. Finally, the fact that the pH of the aqueous phase did not change after extraction is further evidence of the ion-ion paired monomer.

Determination of the True Partition Coefficient.—Earlier it was stated that if the extraction was carried out in an aqueous buffered solution, pH 5.0, the dissociation of species c_1 and c_2 would be more than 99.5%. Another series of experiments were devised to attempt to measure K_i. Should there be association in the aqueous phase, then the true partition coefficient, K_d, could be determined.

From a consideration of the equilibria involved (see the chemical equation) the ionization constant, K_i, and the true partition coefficient, K_d, were expressed as follows

$$K_i = \frac{(BH^+)(Tr^-)}{(BHTr)} \qquad \text{(Eq. 14)}$$

$$K_d = \frac{(BHTr)_s}{(BHTr)_w} \qquad \text{(Eq. 15)}$$

where s refers to the nonpolar solvent and w refers to the water solvent. Consider that the concentration of BH^+ is equal to the concentration of Tr^- in aqueous solution and consider the dissociation to be α, then

$$K_i = \frac{(c_w\alpha)(c_w\alpha)}{c_w(1-\alpha)} \qquad \text{(Eq. 16)}$$

If the concentration of the BHTr in the nonpolar solvent is c_s then

$$K_d = \frac{c_s}{c_w(1-\alpha)} \qquad \text{(Eq. 17)}$$

and

$$c_w(1-\alpha) = \frac{c_s}{K_d} \qquad \text{(Eq. 18)}$$

Substituting Eq. 18 into Eq. 16

$$K_i = \frac{c_w^2\alpha^2 K_d}{c_s} \qquad \text{(Eq. 19)}$$

Solving for α from Eq. 9

$$\alpha^2 = \frac{K_i \cdot c_s}{c_w^2 K_d} \qquad \text{(Eq. 20)}$$

TABLE V.—DIELECTRIC CONSTANTS OF THE NONPOLAR PHASES AND THE pH OF THE AQUEOUS RESIDUE

Solvent	Dielectric Constant	Amine Used	Dielectric Constant of BHTr in Nonpolar Phase	pH of Aqueous Residue
CH_2Cl_2	9.50	di-iso-amylamine	9.70	4.95
	9.50	di-*n*-amylamine	9.70	4.95
	9.50	di-*n*-butylamine	9.75	4.95
	9.50	di-*n*-propylamine	9.65	4.98
$CHCl_3$	5.45	di-iso-amylamine	5.40	4.95
	5.45	methylene blue	5.50	...
$CHCl_3$:CCl_4 (2:1)	4.08	di-iso-amylamine	4.03	4.95
	4.08	di-*n*-butylamine	4.20	...
	4.08	di-*n*-propylamine	4.10	...
$CHCl_3$:CCl_4 (1:1)	3.50	di-iso-amylamine	3.55	4.95

TABLE VI.—DISTRIBUTION OF DI-ISO-AMYLAMINE TROPAEOLINATE BETWEEN METHYLENE CHLORIDE AND WATER[a]

Amine Concn.	Tropaeolin 00 Concn.	c_2[b]	c_w[c]	c_1	c_1c_2	k'[d]	$\frac{\sqrt{c_2}}{c_w}$	$\frac{c_2}{c_w}$
0.6	0.6	0.268	0.314	0.332	0.104	2.58	1.65	0.854
0.8	0.8	0.394	0.366	0.406	0.149	2.65	1.71	1.08
1.0	1.0	0.517	0.448	0.497	0.222	2.33	1.61	1.15
1.2	1.2	0.665	0.481	0.535	0.257	2.59	1.70	1.38
1.4	1.4	0.829	0.561	0.571	0.320	2.59	1.66	1.48
1.6	1.6	0.938	0.632	0.662	0.418	2.24	1.53	1.48
1.8	1.8	1.17	0.674	0.633	0.427	2.73	1.60	1.73

[a] Concentrations are expressed as micromoles per 40 ml. [b] Also referred to as c_2. [c] Also referred to as c_w. [d] The value k' was determined by dividing c_2 by c_1c_2.

$$\alpha = \sqrt{\frac{K_i}{K_d}} \cdot \frac{\sqrt{c_2}}{c_w} \qquad \text{(Eq. 21)}$$

From Eq. 17 another expression for α is obtained

$$K_d - K_d\,\alpha = \frac{c_2}{c_w} \qquad \text{(Eq. 22)}$$

or

$$\alpha = 1 - \frac{1}{K_d} \cdot \frac{c_2}{c_w} \qquad \text{(Eq. 23)}$$

Combining Eqs. 21 and 23, one gets

$$\sqrt{\frac{K_i}{K_d}} \cdot \frac{\sqrt{c_2}}{c_w} = 1 - \frac{1}{K_d} \cdot \frac{c_2}{c_w} \qquad \text{(Eq. 24)}$$

Rearranging Eq. 24

$$\frac{\sqrt{c_2}}{c_w} = \sqrt{\frac{K_d}{K_i}} - \sqrt{\frac{K_d}{K_i}} \cdot \frac{1}{K_d} \cdot \frac{c_2}{c_w} \qquad \text{(Eq. 25)}$$

or in its final form

$$\frac{\sqrt{c_2}}{c_w} = \sqrt{\frac{K_d}{K_i}} - \frac{1}{\sqrt{K_dK_i}} \cdot \frac{c_2}{c_w} \qquad \text{(Eq. 26)}$$

The y intercept is equal to $\sqrt{K_d/K_i}$ and the slope or gradient is equal to $1/\sqrt{K_dK_i}$. Therefore, the ionization constant, K_i, and the true partition coefficient, K_d, can be measured by varying the concentration of both amine and tropaeolin 00 in the aqueous phase and extraction of BHTr. From these studies the values for $\sqrt{c_2}/c_w$ and c_2/c_w can be calculated. The data are then plotted to determine the y intercept and the slope.

Therefore, to determine the ionization constant and true partition coefficient, varying but equal micromolar concentrations of di-iso-amylamine and tropaeolin 00 were mixed, and to this was added an equal volume of methylene chloride. After partitioning, the concentrations c_1, c_2, and c_3 were determined. These values are recorded in Table VI. These data were used to test Eq. 13. The plot in Fig. 4 was developed from the data in Table VI. The data in columns 8 and 9 of Table VI were calculated to see if Eq. 26 could be useful in determining the true partition constant, K_d.

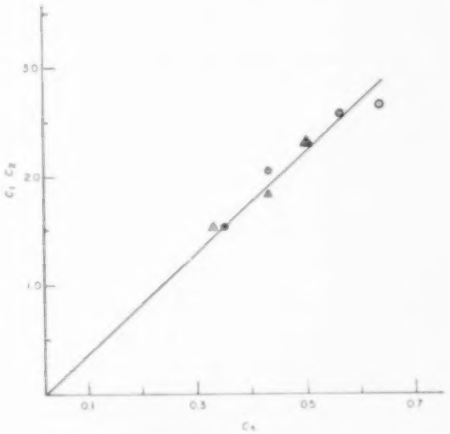

Fig. 3.—The relation of ion concentrations of the solute species in the aqueous phase and the monomer neutral species in the nonpolar phase using di-iso-amylamine tropaeolinate. △, concentration of c_1 held constant; ○, concentration of c_2 held constant.

The data in columns 8 and 9 of Table VI were plotted in Fig. 5. The regression line was determined using the method of least squares (13). The intercept was determined to be 1.767 with a negative slope of 0.1. Equation 26 is an equation for a

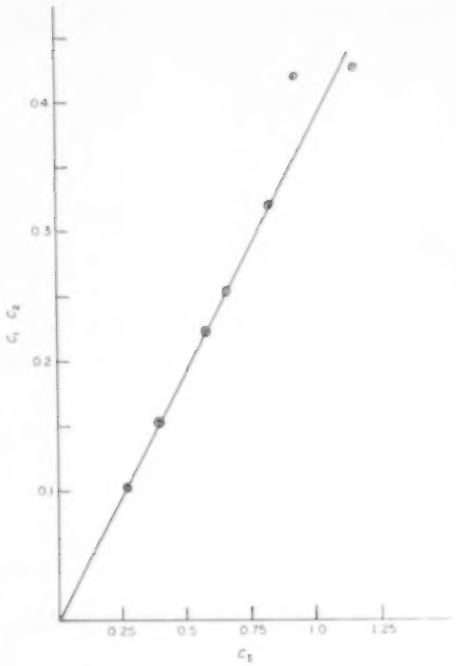

Fig. 4.—The distribution of di-iso-amylamine tropaeolinate between methylene chloride and water.

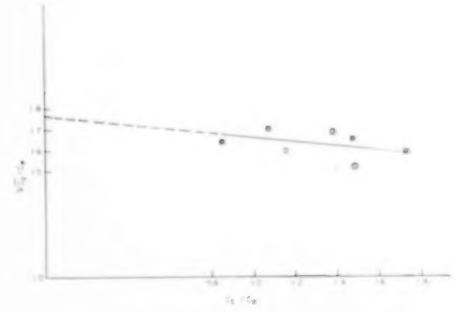

Fig. 5.—The distribution of di-iso-amylamine tropaeolinate between methylene chloride and water at 21°.

straight line. Solving for the intercept and the slope, it was found that K_d was 17.67 and that K_i was 5.66. Considering unit concentration of BHTr and using Eq. 14 and the quadratic equation, it was found that α was 0.867. From Fig. 5, using Eq. 21, it was found that α varied between 0.94 and 0.91. It is concluded, therefore, that if association of the ions in the aqueous solution occurs to a small extent, the true partition or distribution constant, K_d, can be determined.

The amine tropaeolinates were isolated and recrystallized. The melting points were determined and recorded in Table VII. Since the isolated salts have a relatively high melting point, the ion-ion pair should be referred to as a salt and not a loose complex.

TABLE VII.—MELTING POINTS OF SOME AMINE TROPAEOLINATES

Amine Tropaeolinates	M.P., °C. (uncorr.)
Di-n-propylamine tropaeolinate	203–207
Di-n-butylamine tropaeolinate	186–193
Di-n-amylamine tropaeolinate	194–197
Di-iso-propylamine tropaeolinate	206–211
Di-iso-butylamine tropaeolinate	189–193
Di-iso-amylamine tropaeolinate	207–217

CONCLUSIONS

1. A quantitative relationship was established for the apparent distribution constant, K, of some amine tropaeolinates and their molecular weights and structures. It was assumed that the dissociation of the salt in an aqueous solution was 100%. It was shown that the K values varied with different nonpolar solvents.

2. At a pH of 5.0 it was calculated, using the Henderson equation, that the degree of dissociation of the amine and tropaeolin 00 in aqueous solution would be greater than 99%. Assuming 100% dissociation it was determined that the apparent partition coefficient of di-iso-amylamine tropaeolinate between methylene chloride and water was 102.8. At the same pH it was determined experimentally that dissociation was of the order of 90%. It was then determined that the true partition coefficient for di-iso-amylamine tropaeolinate between methylene chloride and water was 17.67.

3. Using thermodynamic considerations, equations were derived which fitted the numerical data and graphical plots.

4. The melting points of the different amine tropaeolinates are recorded.

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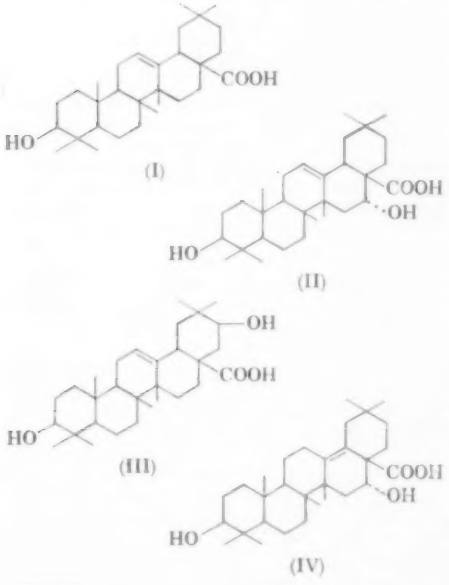
Saponins and Sapogenins VII

Acid Sapogenins Isolated from Maharashtrian *Albizzia odoratissima* Seeds

By I. P. VARSHNEY and M. S. Y. KHAN

The saponin obtained from the seeds of *Albizzia odoratissima* Benth from Maharashtra state on hydrolysis gives two triterpenic acids. The acid m. p. 266–270°, acetate m. p. 260–265°, acetyl methyl ester m. p. 279–280°, and methyl ester m. p. 224–225° has been identified as machaerinic acid (3β,21β-dihydroxy-Δ¹²-18-β-oleanene-28-oic acid) by mixed melting point and infrared spectra with authentic sample. The acetate of the second acid genin melted at 228–234°.

ALBIZZIA ODORATISSIMA Benth locally known as "Kala Sisis" is a member of the family *Leguminosae* subfamily *Mimosideae*. It grows wild in certain parts of India. The fixed oil isolated from the seeds of this plant from Uttar Pradesh has been studied earlier in these laboratories for the fatty acid composition (1). No report on the saponin and sapogenin contents of this variety could be found in the literature. During the course of our work on saponins and sapogenins, the family *Leguminoseae* has been found to be a rich source of triterpenic saponins and sapogenins. *A. lebbek* (2, 3) has been found to contain oleanolic acid (I) and echinocystic acid (II), while *A. anthelmintica* (4) yields echinocystic acid (II) alone. *A. procera* (5) gives machaerinic acid (III). All these acids belong to the β-amyrin group. Very recently Barua and Raman (6) studying *A. lebbek* from Bengal reported the isolation of albigenic acid, an isomer of echinocystic acid (IV), in addition to the echinocystic acid (II) and oleanolic acid (I). Repeated attempts to isolate the albigenic acid (IV) from the *A. lebbek* Benth seeds obtained from the states of Uttar Pradesh and Madhya Pradesh were negative. It may be possible that either these varieties do not contain albigenic acid or it may have been a result of isomerization taking place in the course of hydrolysis by hydrochloric acid and needs further substantiation.



As the preliminary tests for the saponin in *A. odoratissima* seeds obtained from Silviculturist, Poona, Maharashtra, showed the presence of a considerable amount of saponin, it was thought desirable to investigate these seeds for the saponin and sapogenin contents.

A quantity of well powdered defatted seeds were exhausted with ethanol. The recovery of the solvent left an oily residue from which a light cream colored hygroscopic powder which gave all the tests for saponin (7) was obtained in the usual manner.

The saponin solution, on hydrolysis with sulfuric acid, deposited a colorless precipitate of the genin which was filtered and washed free of sulfuric acid with water.

The genin thus obtained was purified by formation of a potassium salt. The potassium salt solution, after ether extraction to remove any neutral material if present, was acidified with hydrochloric acid which precipitated the acid genin. The acid genin was filtered and washed with water until the washings were neutral. The acid genin was acetylated with pyridine and acetic anhydride in the cold. The acetate obtained was crystallized from methanol containing a few drops of chloroform. It gave two

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acetates quite different from each other as colorless, fine needles, m. p. 260–265° and m. p. 228–234°. Analysis of the acetate m. p. 260–265° showed the formation of a diacetate corresponding to the formula $C_{34}H_{52}O_6$. The diacetate gave a positive test for a double bond with tetranitromethane.

The diacetate m. p. 260–265°, on deacetylation with methanolic potassium hydroxide, yielded the acid genin, m. p. 266–270°. Analysis of the genin indicated the presence of two hydroxyl groups and one carboxyl group in the pentacyclic triterpenic compound. The infrared spectra of the acetate and the genin are consistent with this formulation and resembled the spectra of the triterpenic acids of β-amyrin group (2). It showed the absorption between 11.8–12.4 μ, indicative of the presence of a triply substituted ethylenic linkage [*cf.* Barton, *et al.* (8), King, *et al.* (9), Ruzicka, *et al.* (10)]. The genin gave a positive Liebermann-Burchard reaction and all the color tests of triterpenes. The tetranitromethane test was positive, confirming the presence of at least one carbon-carbon double bond. This acid being virtually insoluble in a solution of sodium bicarbonate showed its weak acid nature. The ease of the acetylation and saponification of all the two acetyl groups suggested that the two hydroxyl groups were either equatorially oriented secondary and/or primary alcoholic functions.

The carboxyl function was readily detected and fixed by the formation of a methyl ester, m. p. 224–225°, on treatment of the acid with diazomethane. The diacetate also, on methylation with diazomethane, gave a diacetyl methyl ester, m. p. 279–280°. Analysis of the diacetyl methyl ester showed the genin to be a monocarboxylic acid.

The position of the double bond with respect to the carboxyl group was deduced by the ready formation of a highly crystalline acetyl bromolactone, m. p. 288–290°, through bromine in acetic acid and diacetate. This also showed the compound to be either βγ or γδ unsaturated acid. The easy formation of the bromo-lactone in very good yield gave further support to the olene-12-ene (β-amyrin) structure for the acid [*cf.* King, *et al.* (9, 11); Jeger (12)]. Therefore, it has been inferred that the acid is unsaturated in position −12 with a carboxyl group in position −28.

The comparison of the physical constants of the genin and all its derivatives with all the known acids of the β-amyrin-oleanolic acid group containing two hydroxyl groups indicated that the present acid is identical with machaerinic acid (3β,21β-dihydroxy-Δ^{12}-18β-oleanene-28-oic acid) (III) earlier isolated from *A. procera* seeds (5). The mixed melting points of all the derivatives and the genin with authentic samples of machaerinic acid and derivatives and the infrared spectra of the acetyl methyl ester with acetyl methyl machaerinate (Fig. 1) which are superimposable confirmed its identity without any doubt.

Further work on the second acid genin (acetate m. p. 228–234°) will be taken up as soon as a sufficient quantity of the genin has been collected.

EXPERIMENTAL

All the melting points recorded in this paper have been taken on Kofler's hot microscopical stage and are corrected. The infrared spectra have been taken in these laboratories by one of the authors (I. P. V.) using a Perkin-Elmer spectrometer, Infracord model 137 in Nujol mull. The microanalyses are by Weiler and Strauss, Oxford, England.

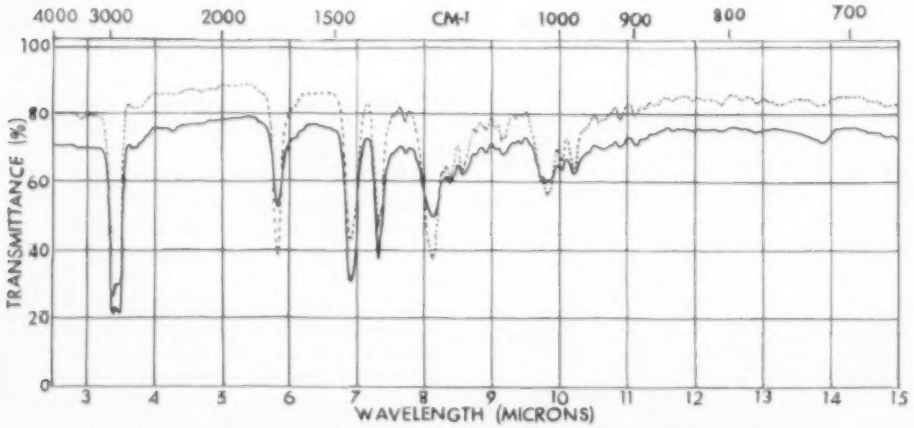

Fig. 1.—Infrared spectra of the acetyl methyl ester and acetyl methyl machaerinate.

Defatting.—Well powdered seeds (250 Gm.) were exhausted in a Soxhlet apparatus with light petroleum ether (40–60°). The recovery of the solvent gave a greenish-yellow oil.

Extraction.—The defatted seed powder was exhausted with 95% alcohol in a Soxhlet apparatus, and after the solvent was recovered a brown, syrupy liquid remained. It was extracted successively with petroleum ether, ether, carbon tetrachloride, chloroform, and acetone. The residue was dissolved in ethyl alcohol and filtered. The solution was added, dropwise, to a large amount of ether when a light brown precipitate was obtained. This operation of solution in alcohol and precipitation was repeated several times, and finally the precipitation was made with acetone. The final precipitate was taken up in alcohol and treated with activated charcoal. It was again precipitated with acetone. This gave a light cream colored, hygroscopic powder of saponin. This product gave all the tests for saponins, such as sternutatory action, foaming with water, toxicity to fish in low concentrations, and specific color reactions.

Isolation of Sapogenin.—The cream colored powder of the saponin obtained by the ether/acetone precipitations was dissolved in a large amount of water and hydrolyzed with sulfuric acid (10%) by heating the solution first on a boiling water bath for one hour and then completing the hydrolysis by refluxing for another hour. After completion of the hydrolysis, the precipitate was filtered, washed with water until the washings were neutral, and finally dried. The genin thus obtained was dissolved in alcohol and decolorized with activated charcoal.

The crude genin was converted into the potassium salt by heating with alcoholic solution of caustic potash for an hour and thereafter distilling off half the solvent. The solution was diluted with a large quantity of water and extracted three times with ether. The ethereal extracts were combined and washed free of alkali. Recovery of the solvent did not leave an appreciable quantity of neutral sapogenin. The alkaline solution left after ether extraction was acidified with hydrochloric acid and, after two hours, the precipitate obtained was filtered, washed with water until free of hydrochloric acid, and dried. This gave the acid genin.

Acetylation.—The acid genin was acetylated with acetic anhydride in the presence of pyridine by keeping the mixture overnight. It was poured into ice water when the acetate was precipitated and filtered. The precipitate, after washing free of acid and pyridine, was fractionally crystallized from methanol into two different products which were separated. Their melting ranges were 260–265° and 228–234°. They both gave positive reactions with tetranitromethane.

Acetate m. p. 260–265°.—Mixed melting point with diacetyl machaerinic acid 260–264° (*cf.* machaerinic acid diacetate m. p. 258–260°) (5).

Anal.—Calcd. for $C_{34}H_{52}O_8$: C, 73.35; H, 9.41. Found: C, 73.62; H, 9.66. Infrared spectra: ν $Nujol_{max.}$ 1,723, 1,689, 1,257 cm.$^{-1}$.

Deacetylation.—The acetate m. p. 260–265° (300 mg.) was refluxed for two hours with methanolic potassium hydroxide (150 ml. 5%). The solution was diluted with a large amount of water and left overnight at room temperature but no solid potassium salt separated. The solution was acidified with hydrochloric acid which precipitated the acid sapogenin. The precipitate was filtered, washed with water until free of acid, and crystallized from methanol in needles, m. p. 266–270°. It gave a yellow color with tetranitromethane and a positive Liebermann-Burchard reaction. It showed no depression in melting point when mixed with machaerinic acid [*cf.* machaerinic acid m. p. 256–258° (5)].

Anal.—Calcd. for $C_{30}H_{48}O_4$: C, 76.22; H, 10.23. Found: C, 76.19; H, 10.37. Infrared spectra: ν $Nujol_{max.}$ 3,400, 3,240, 1,741, 1,689 cm.$^{-1}$.

Methylation.—The acid genin (100 mg.) was dissolved in ether and an excess of the ethereal solution of the diazomethane was added to it. The solution was left overnight and the excess of the diazomethane was then removed on a water bath. The residue left after evaporation of the ether was crystallized from methanol in needles, m. p. 224–225°. This showed unsaturation with tetranitromethane. Mixed melting point with methyl machaerinate 223–225° [*cf.* machaerinic acid methyl ester, m. p. 224–225° (5)].

Infrared spectra: $\nu$$Nujol_{max.}$ 3,400, 1,724 cm.$^{-1}$.

Acetyl Methyl Ester.—The acetate (100 mg.) in ether solution was methylated with an ethereal solution of diazomethane. After twenty-four hours contact, the ether was evaporated and the product crystallized from methanol as colorless fine needles, m. p. 279–280°. It gave a positive reaction with tetranitromethane. Mixed melting point with diacetyl methyl machaerinate 279–280° [*cf.* diacetyl methyl machaerinate, m. p. 278–280° (5)].

Anal.—Calcd. for $C_{35}H_{54}O_8$: C, 73.64; H, 9.53. Found: C, 72.96; H, 9.39. Infrared spectra: Fig. 1.

Acetyl Bromolactone.—The acetate (150 mg.) and 300 mg. of sodium acetate were dissolved in acetic acid (25 ml., 90%), and 6 ml. of a 4% solution of bromine in acetic acid was added, dropwise. The mixture was kept for an hour at room temperature and then was poured into 300 ml. of water containing 500 mg. of sodium thiosulfate. The precipitate that formed was filtered, washed with water, and crystallized from methanol as colorless crystals, m. p. 288–290°. It gave no yellow color with tetranitromethane.

Anal.—Calcd. for $C_{34}H_{51}BrO_8$: C, 64.23; H, 8.08. Found: C, 64.09; H, 8.25.

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Direct Determination of Niacinamide in Multivitamin Preparations

By O. PELLETIER and I. A. CAMPBELL

A simple method for the direct determination of niacinamide in pharmaceutical preparations is described. The method involves the reaction of niacinamide in potassium dihydrogen phosphate with cyanogen bromide and barbituric acid. The resulting purple color has a maximum absorbance at 550 mμ. Niacin does not interfere in the reaction unless present at about three times the concentration of that of the amide. The method is accurate and reproducible. Results on multivitamin preparations compare favorably with those by the A.O.A.C. procedure and recoveries of added niacinamide are quantitative.

NIACINAMIDE rather than niacin is generally used in multivitamin preparations because of the possible undesirable side effects of the latter. Although the chemical estimation of total niacin-niacinamide after hydrolysis is relatively simple (1, 2), the methods are not specific for the amide. Chromatographic methods (3, 4) for the differentiation of the two compounds are time-consuming and other methods (5–9) are too complex for routine use. There is obviously a need for a specific method for the direct estimation of niacinamide.

An approach to this problem was made by Lisboa (10) who demonstrated that niacin and niacinamide in Sorensen's buffer at pH 7.2 reacted differently with barbituric acid and cyanogen bromide. He determined niacinamide directly in the presence of niacin by measuring the resulting absorbance at 513 mμ. Preliminary tests of Lisboa's method indicated that it lacked sensitivity and did not follow Beer's law at higher concentrations. This paper describes factors affecting the reaction of niacinamide with cyanogen bromide and barbituric acid and the applicability of this reaction to the direct estimation of niacinamide in complex multivitamin preparations.

METHOD

Apparatus.—Suitable photoelectric colorimeter or spectrophotometer.

Reagents.—*Cyanogen bromide solution,* 10%, prepare and use under hood; do not let cyanogen bromide or solution come in contact with skin; dissolve a weighed amount of cyanogen bromide in warm water at about 40°; allow to come to room temperature and dilute to 10% solution; store in refrigerator and bring to room temperature before using. *Potassium dihydrogen phosphate,* 3% (KH_2PO_4); dissolve 30 Gm. in water to make 1,000 ml. *Potassium dihydrogen phosphate,* 0.3%; prepared by diluting 3% KH_2PO_4 1 to 10 with water. *Barbituric acid buffered solution, saturated,* (2%); prepare volume required for each batch of assays by adding 100 ml. of 3% KH_2PO_4 to each 2 Gm. of reagent grade barbituric acid; let stand for one hour with occasional shaking; filter before using.

Niacinamide Standard Solutions.—*Stock Solution.*—Weigh 50 mg. U. S. P. Nicinamide Reference Standard, dissolve in 60% ethyl alcohol, and make up to 200 ml. Store in refrigerator.

Working Standard Solution.—Pipet 2 ml. of stock solution into a 100-ml. volumetric flask and make to volume with 0.3% KH_2PO_4. This solution contains 5 mcg./ml.

Assay Solution.—Place a measured amount of sample in a flask and add a volume of 0.3% KH_2PO_4 equal in ml. to at least twice the amount of niacinamide in mg. Tablets should be ground first. For capsules, add about 2 ml. of ethylene dichloride to aid in dispersion. If sample is not readily soluble, shake and heat so that it may be evenly dispersed. Dilute to about 5 mcg. per ml. with 0.3% KH_2PO_4. Filter if necessary.

Reaction.—To 1 ml. of dilute standard or assay solution in colorimeter tube, add 0.5 ml. cyanogen bromide, mix, stopper, and let stand twenty-five to thirty minutes. Add 10 ml. barbituric acid and mix by swirling. (If barbituric acid cannot be added at this time, transfer tubes to a bath of crushed ice to stabilize cyanogen bromide reaction.) Set spectrophotometer at zero absorbance at 550 mμ with a standard blank in which cyanogen bromide is replaced by water and measure maximum absorbance of reaction product in two to four minutes after addition of barbituric acid. Prepare sample blanks by replacing cyanogen bromide with water.

Calculations.—Calculate on basis of aliquots taken as follows

$$\text{mg. per unit sample} = \frac{Au}{As} \times \frac{5}{1{,}000} \times \text{dilution factor}$$

where Au = corrected absorbance of unknown and As = absorbance of standard.

EXPERIMENTAL RESULTS

Selection of Extracting Medium.—Hydrolysis of niacinamide occurred in most extracting solutions tested, including very dilute solutions of acid and solutions of dibasic and tribasic phosphate. However, KH_2PO_4 did not cause hydrolysis and therefore appeared to be the most suitable extractant. It was found that maximum absorbance was produced with a concentration of about 0.3% KH_2PO_4.

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Table I.—Per Cent Recovery of Niacinamide from Niacin-Niacinamide Mixtures

Niacinamide, %	Recovery, % 5 mcg./ml. Test 1	5 mcg./ml. Test 2	10 mcg./ml. Test 1	10 mcg./ml. Test 2
95	100.5	99.5	100.0	99.9
75	100.0	99.5	100.0	100.0
50	101.0	100.5	100.5	100.7
25	103.5	101.0	102.1	102.4
5	111.6	107.5	109.2	111.4

Factors Affecting the Reaction.—Color development is dependent on both the reaction of niacinamide with cyanogen bromide and on the coupling with barbituric acid. The effect of various factors on these two reactions was determined using 10 mcg. niacinamide in 1 ml. solution. Concentrations and volumes of reagents and time and temperature reaction influenced the amount of color formed. The optimum amount of cyanogen bromide was found to be about 0.5 ml. The cyanogen bromide reaction was complete in about twenty-five minutes at toom temperature and remained stable for about five minutes. By cooling in ice water at that time, the stability could be maintained for over one hour. For color development no advantage was gained by using an amount of more than 10.0 ml. or a concentration of more than 2% barbituric acid. The optimum concentration of KH_2PO_4 as a buffer for barbituric acid was 3%.

Concentration of Niacinamide.—There was a linear relation between concentration and absorbance up to 10 to 12.5 mcg. niacinamide per ml. depending on the instrument used. The most suitable concentration to use appeared to be 5 or 10 mcg.

Effect of Niacin on the Determination of Niacinamide.—Mixed solutions of niacin and niacinamide containing 5 to 95% niacin were assayed at the 5 and 10-mcg. levels of niacinamide and results are shown in Table I. There was no significant interference of niacin up to the 50% level. Results were 2.3% higher at the 75% level and about 10% higher at the 95% level where the solutions contained almost 20 times as much niacin as niacinamide.

Assays and Recoveries of Niacinamide in Pharmaceutical Products.—The proposed method was compared with the A.O.A.C. method (1) in assays and in recovery tests on 13 representative pharmaceutical products. The tablets were ground to a fine powder and well mixed. Capsules were opened and their contents uniformly mixed. Duplicate samples were weighed out. In one, niacinamide was determined directly; to the other, niacinamide was added in an amount equivalent to label claim and then assayed. Results are reported in Table II in terms of per cent of label claim or per cent recovery of added niacinamide. In the assay of samples, the difference between the two methods was small except for a liver extract elixir and a syrup preparation which gave lower values by the A.O.A.C. method. These two samples became extremely dark upon hydrolysis and yielded very high blanks by the A.O.A.C. method. Excluding these two products the mean difference between the two methods was 0.2% and in the recovery test a mean value of 99.6 ± 3.0% was obtained with the A.O.A.C. method, while the proposed method yielded a mean recovery of 100.2 ± 1.4%.

Reproducibility of Assays.—To test and compare the reproducibility of assays by both methods, an elixir vitamin B-compound preparation containing yeast extract was assayed on five different days and results are reported in Table III in terms of per cent of label claim. Values of 101.3 ± 1.2% by the proposed method compared favorably with 102.2 ± 2.5% by the A.O.A.C. method.

Specificity of Reaction.—To test the specificity of

Table III.—Results of Repeated Assays of Niacinamide in A Vitamin B Compound-Yeast Elixir Preparation By Proposed Method and A.O.A.C. Method

Day	Per Cent of Label Claim, 1.25 mg./ml. Proposed Method	A.O.A.C. Method
1	103.0	104.7
2	102.5	98.2
3	100.0	99.2
4	100.6	102.1
5	100.5	102.2
Mean	101.3	102.2
S. D.	1.2	2.5

Table II.—Assay and Recovery of Niacinamide in Multivitamin Preparations By Proposed Method and A.O.A.C. Method

Multivitamin Preparations	Label Content mg./Tab., Capsule, ml.	Label Claim, % A.O.A.C.	Label Claim, % Proposed	Difference	Recovery, %[b] A.O.A.C.	Recovery, %[b] Proposed
Tablets	20	100.0	101.0	+1.0	100.9	101.0
Tablets	25	103.1	100.0	−3.1	94.1	102.0
Capsules	100	100.0	102.0	+2.0	99.7	99.0
Capsules	150	103.3	98.9	−4.4	99.1	99.6
Tablets (minerals)	2.5	111.9	112.3	+1.4	101.3	103.7
Capsules (minerals)	10	115.4	114.5	−0.9	98.7	99.5
Capsules (minerals)	10	108.3	107.6	−0.7	99.2	97.7
Tablets (minerals)	20	92.0	95.0	+3.0	99.5	100.0
Tablets (minerals)	50	94.6	95.3	+0.7	97.0	100.4
Injectable	100	99.2	100.0	+0.8	99.2	100.0
Elixir (yeast)	1.25	104.7	103.0	−1.7	107.2	100.0
Elixir (liver)	1.25	101.8	114.7	(+12.9)[a]	(104.7)	100.6
Syrup (minerals)	1.0	91.0	115.0	(+24.0)	(89.0)	99.0
Mean	...	...	...	−0.2	99.6	100.2
S. D.	...	...	...	...	3.0	1.4

[a] Values in parenthesis not included in mean and S. D. [b] Recovery of an amount added equivalent to labeled claim.

TABLE IV.—REACTION OF SOME PYRIDINE DERIVATIVES IN PROPOSED METHOD AND IN A.O.A.C. METHOD

Derivatives	Color Formed in Proposed Reaction at Concentration of		Per Cent Absorbance Comparison with Niacinamide[a]	
	10 mcg.	100 mcg.	Proposed Method	A.O.A.C. Method
α-Substituted				
Picolinic acid	nil	nil	0	0
α-Picoline	nil	nil	0	3
2,4-Lutidine	nil	nil	0	0
β-Substituted				
Niacin	light yellow	light yellow	0	99
Niacinamide	purple	purple	100	100
Niacinamide HCl	purple	purple	100	97
Nicotinic acid ethyl ester	purple	purple to red	101	121
Nicotinic acid dimethyl amide	purple	red-purple	69	102
Nicotinic acid diethyl amide	salmon pink	orange to red	16	241
Pyridine-3-sulfonic acid	salmon pink	salmon pink	7	36
Nicotinic nitrile	trace pink	reddish	6	65
Nicotinuric acid	nil	trace pink	2	6
Nicotine	yellowish	brownish	1	9
γ-Substituted				
Isonicotinic acid	nil	nil	0	3
Isonicotinic acid amide	blue	blue	25	0
Isonicotinic acid hydrazide	greenish	greenish	2	2
N'-Substituted				
Trigonelline	nil	nil	0	0
N'-Methyl nicotinamide	nil	light purple	2	3
Miscellaneous				
Pyridoxine HCl	nil	nil	0	0
Pyridoxamine 2 HCl	nil	nil	0	0
Pyridine	light yellow	light purple	1	40
α,α'-Dipyridyl	nil	nil	0	2

[a] Per cent absorbance was calculated on an equimolar basis, using concentrations of 10 mcg. for the proposed method and 5 mcg. for the A.O.A.C. method.

the method, several pyridine derivatives were assayed at 10 and 100 mcg. per test tube. Results are shown in Table IV. The α-substituted derivatives gave no reaction. The β-substituted derivatives varied widely in the color produced but only nicotinic acid ethyl ester and dimethyl amide gave the purple color characteristic of niacinamide. The γ-substituted compounds yielded blue, green, or no color. *N'*-Methyl nicotinamide produced a purple color only at relatively high concentrations. Pyridine produced a more stable color at higher concentrations. No reaction was given by the two vitamin B_6 compounds. Data on the relative specificity of the two reactions indicate that for the compounds listed the proposed method is at least as specific as the A.O.A.C. method.

DISCUSSION

The proposed method differs from that originally presented by Lisboa in extracting agent, pH of reaction, amounts of reagents, order of adding reagents, and absorbance maximum of reaction. Based on the absorbance per mg. of niacinamide, the proposed was about 10 times as sensitive as the former procedure. Interference of niacin in the recovery of niacinamide was evident only when niacin was present in three times the amount of niacinamide, but even at that level the effect was not serious. Examination of results published by Lisboa suggested greater interference of niacin at about the same proportions. Commercial preparations containing the amide ordinarily have little, if any, niacin. Assays and recovery tests with representative preparations indicated that the proposed method compared favorably with the A.O.A.C. method. The proposed method was highly reproducible and was applicable to all preparations while the A.O.A.C. method gave unsatisfactory results with a syrup and a liver elixir preparation because of interfering colors produced by hydrolysis. In the proposed method sample blanks were often not necessary because dilute assay solutions were clear and contained no interfering color.

The reaction with barbituric acid is much more specific for the determination of the amide than the previous methods using hydrolysis. Of the several compounds tested, only nicotinic acid ethyl ester and nicotinic acid dimethyl amide reacted similarly to niacinamide, but their presence would not be expected in multivitamin preparations.

The time required for assay by the proposed method was shorter than by the A.O.A.C. method since hydrolysis and pH adjustments were not required. The main advantages of the proposed method are that it determines niacinamide directly and is simpler than available methods.

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Study of the Cationic Exchange Properties of Acid Activated Bentonite I

Effect of Structure Variation, pH, and Ionic Strength on the Exchange Rates of a Series of β-Phenylethylamines

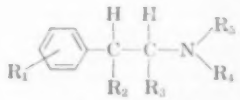
By EARL W. SEUGLING, Jr.†, and EARL P. GUTH

Acid activated bentonite was prepared. The exchange isotherms of a series of β-phenylethylamines were determined. A modified spectrophotometric assay was developed to determine the amount of amine in the presence of irrelevant absorbing substances. The effect of time, particle size of the exchanger, structural variation of the amines, pH, and ionic strength were studied. The extent of exchange and adsorption was estimated. The exchange rate increased with alkylation of the amino group. The position of the hydroxyl group on the amine structure affected the exchange rate. Decreasing the pH led to a decrease in the exchange rate, and increasing the pH led to an increase in the adsorption rate. The amount of amine exchanged at high concentration decreased with ionic strength. The nature of the electrolyte cation influenced the amount of amine exchanged.

THE CATIONIC exchange activity of acid activated bentonite has been reported in earlier studies. Gorman and Guth (1) investigated the exchange properties of acid activated bentonite with application to the assay of pharmaceuticals. Young and Guth (2) continued this study comparing acid activated American and Italian bentonite. Huber and Guth (3) studied the exchange properties of natural American and Italian bentonite. Although these studies indicated certain qualitative aspects of the exchange properties of these clays, they were not sufficiently conclusive to derive quantitative relationships.

It was the purpose of this investigation to determine the effect of structure variation, pH, and ionic strength of the contact solution on the amount of amine exchanged or adsorbed, and, also, to determine the extent of exchange and adsorption. A series of β-phenylethylamines was selected with variations of alkylation of the amino group and the position of the hydroxyl group on the basic structure. The amines used in this study are listed in Fig. 1.

Since acid activated bentonite has hydrogen ion as its principal exchangeable cation, the saturation of exchange sites by other cations would be indicated by no further change in the pH of the equilibrium solution. The removal of organic cations from solution would be by an adsorptive mechanism. The comparison of the exchange capacity of inorganic and organic cations, as indicated by constant pH of the equilibrium solution, indicates the extent of adsorption.



Amine	R_1	R_2	R_3	R_4	R_5
Phenylephrine HCl[a]	m-OH	OH	H	CH_3	H
Hydroxyamphetamine HBr[b]	p-OH	H	CH_3	H	H
Phenylpropanolamine HCl[c]	H	OH	CH_3	H	H
Ephedrine HCl	H	OH	CH_3	CH_3	H
1-N-Ethylephedrine HCl[d]	H	OH	CH_3	C_2H_5	CH_3
Amphetamine sulfate	H	H	CH_2	H	H
Methamphetamine HCl[e]	H	H	CH_3	CH_3	H

These drugs were supplied by the manufacturers, respectively, as: [a] Neosynephrine HCl, [b] Paredrine HBr, [c] Propadrine HCl, [d] Nethamine HCl, [e] Methedrine HCl.

Fig. 1.—Amine series.

EXPERIMENTAL

Preparation of Acid Activated Bentonite.—According to Lamar (4) acid activation increases the adsorptive properties of bentonite. In addition, the exchange sites are occupied completely by hydrogen ions, which insures a more uniform exchange reaction. Acid activated bentonite was prepared by a method similar to that used by Gorman (1) with the following modifications: the mixture was allowed to macerate for thirty days; the bentonite[1] was washed acid-free with distilled water, the washing was continued until the pH of the filtrate remained constant at 5.8. It was noticed that filtrates which

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[1] BC Volclay (American Colloid Co.).

gave a negative sulfate test with barium chloride had pH values as low as 3.0, indicating the presence of free acid. Subsequent comparison of the exchange capacities of fractions washed to constant pH and those washed sulfate-free showed that the latter have an exchange capacity 96% of the former.

The sediment from the pH constant suspension was collected and dried. The resulting hard lumps were ground in a micropulverizer, and the powder was classified according to particle size.

Evaluation of Exchange Properties.—The method used to evaluate the exchange properties of acid activated bentonite followed the usual procedure (5). Unless otherwise specified, 100 mg. of bentonite was weighed in a 40-ml. vial. A 10-ml. aliquot of the test solution was pipetted into the vial, and the vial stoppered. The vials were placed in a wrist action shaker for a fifteen-minute period. The coarse suspension of the bentonite and test solution was filtered using the first portion of the filtrate to thoroughly rinse the vial. The filtrate was assayed spectrophotometrically. The ratio of the solution volume to the weight of bentonite was kept constant, for this has an effect on exchange (1).

Modified Assay Procedure.—Acid activation of the bentonite results in a partial destruction of the silicate lattice which, upon use, yields a colloidal suspension of these decomposition products. Filtration or centrifugation does not remove these substances. In the ultraviolet range where the amines absorb, these substances also absorb and increase the extinction of the amine. The amount of interfering substance present is not constant but is a function of the amount of amine exchanged by the bentonite. Therefore, a method was devised to measure the amount of amine in solution in the presence of other solutes or absorbing substances.

This method was developed following the approach of Morton and Stubbs in their analysis of vitamin A oils (6). This method of correction for unknown irrelevant absorption is based on accurate measurements of intensities of absorption at three wavelengths, not far apart, and the assumption that the irrelevant absorption is linear over the wavelength range. At three wavelengths, L_1, L_2, and L_3, the extinction for the pure amine would be E_{L_1}, E_{L_2}, and E_{L_3}, where $E = \log I_0/I$. If E_1, E_2, and E_3 are the observed values with unknown absorbing substance, and x_1, x_2, and x_3 are the contributions of unknown absorption, then it follows from Fig. 2 that $E_1 - x = E_{L_1}$, $E_2 - x_2 = E_{L_2}$, and $E_3 - x_3 = E_{L_3}$. Also, the ratios $k_1 = E_{L_1}/E_{L_2}$ and $k_2 = E_{L_3}/E_{L_2}$ would be valid, and it follows that

$$k_1 = \frac{E_1 - x_1}{E_2 - x_2} \qquad \text{(Eq. 1a)}$$

and

$$k_2 = \frac{E_3 - x_3}{E_2 - x_2} \qquad \text{(Eq. 1b)}$$

Since the values, x_1, x_2, x_3 lie on a straight line, equations may be derived for these values. Substituting the equations for x_1 and x_3 in Eqs. 2a and 2b

$$k_1 = \frac{E_1 - x_2 - m(L_1 - L_2)}{E_2 - x_2} \qquad \text{(Eq. 2a)}$$

where m = slope of straight line $x_1x_2x_3$.

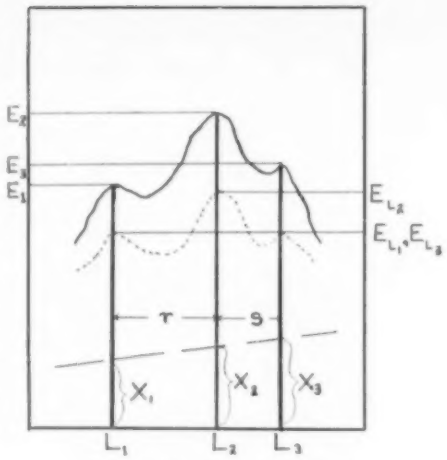

Fig. 2.—Absorption interference.

$$k_2 = \frac{E_3 - x_2 + m(L_2 - L_3)}{E_2 - x_2} \qquad \text{(Eq. 2b)}$$

Let $r = L_1 - L_2$ and $s = L_2 - L_3$, then substituting and rearranging will give Eqs. 3a and 3b

$$m(r) = k_1(x_2 - E_2) + E_1 - x_2 \qquad \text{(Eq. 3a)}$$

$$m(s) = k_2(E_2 - x_2) - E_3 + x_2 \qquad \text{(Eq. 3b)}$$

Dividing Eq. 3a by 3b

$$\frac{r}{s} = \frac{k_1(x_2 - E_2) + E_1 - x_2}{k_2(E_2 - x_2) - E_3 + x_2}$$

and rearranging

$$(rk_2E_2) - (rk_2x_2) - (rE_3) + (rx_2) = (sk_1x_2) - (sk_1E_2) + (sE_1) - (sx_2)$$

Solving for x_2 will give

$$x_2 = \frac{r(E_3 - k_2E_2) + s(E_1 - k_1E_2)}{r(1 - k_2) + s(1 - k_1)} \qquad \text{(Eq. 4)}$$

Thus, if $E_{corrected} = E_{L_2} = E_2 - x_2$, substitution of Eq. 4 for x_2 and simplifying will give Eq. 5

$$E_{corr.} = \frac{r(E_2 - E_3) + s(E_2 - E_1)}{r(1 - k_2) + s(1 - k_1)} \qquad \text{(Eq. 5)}$$

If r and s are chosen so that $k_1 = k_2 = k$, in other words $E_{L_1} = E_{L_3}$, Eq. 5 simplifies to

$$E_{corr.} = a\,E_1 + b\,E_2 \qquad \text{(Eq. 6)}$$

where $a = 1/(1 - k)\ r/(r + s)$, $b = 1/(1 - k)\ s/(r + s)$, $E_1 = E_2 - E_3$, $E_2 = E_2 - E_1$.

The absorption spectra for the amine series were determined using a Perkin-Elmer Spectracord. The irrelevant absorption was found to be linear over the wavelength range of maximum absorption for the amines. The constants for the equation were calculated for each amine, Table I.

The test solutions were analyzed spectrophotometrically using a Beckman model DU spectrophotometer. Distilled water was used as the blank solution unless otherwise specified. The extinction was measured at the specified wavelengths, the E_i

TABLE I.—AMINE SERIES WAVELENGTHS AND CONSTANTS FOR ASSAY

Amine	Wavelength L_1	Wavelength L_2	Wavelength L_3	a	b
Phenylephrine HCl	279.0	272.0	269.0	6.140	2.632
Hydroxyamphetamine HBr	281.0	274.0	269.5	3.854	2.475
Phenylpropanolamine HCl	262.5	256.0	250.5	2.464	2.082
Ephedrine HCl	262.5	256.5	250.5	2.263	2.263
Amphetamine sulfate	263.0	257.0	252.5	2.472	1.857
Methamphetamine HCl	263.0	257.0	252.5	2.414	1.814
l-N-Ethylephedrine HCl	262.5	256.5	251.0	2.405	2.203

and E_2 values were obtained, and then the $E_{corr.}$ value calculated. The concentration of amine was calculated from the formula

$$C = \frac{E_{corr.}}{E_{L_2}{}^{1\%}}$$

where $E_{L_2}{}^{1\%}$ is the meq. extinction coefficient and is equal to the extinction over the product of the length of absorbing material in centimeters (1.000 in this case) times the concentration of amine in milliequivalents per 100 ml. solution.

The assay equation was subjected to an error analysis. The errors in experimental measurement were estimated, and the effect of these errors on the calculated coefficients and final results was evaluated. For example, the $E_{corr.}$ and $E_{L_2}{}^{1\%}$ values for a phenylpropanolamine HCl calculation were

$$E_{corr.} = 0.1690 \pm 0.0055$$

$$E_{L_2}{}^{1\%} = 1.868 \pm 0.065$$

The calculation of concentration would give a value with the following error

$$C = \frac{E_{corr.}}{E_{L_2}{}^{1\%}} = \frac{0.1690 \pm 0.0055}{1.868 \pm 0.065} = 0.0906 \pm 0.0043$$

This value calculated as the per cent deviation is $\pm 4.75\%$.

Several tests were performed to determine the reliability of the modified assay. Solutions of an amine were mixed with the filtrate of a bentonite suspension in order to test the accuracy of the modified assay. The absorption intensity of the amine solution plus the bentonite filtrate was determined and the amount of amine in solution was calculated using the modified assay equation. The calculated values were compared to the known; the per cent deviation of the observed values from the known amounts was ± 0.95.

Dialysis studies were conducted to compare the normal method of bringing the amine solution into contact with the bentonite with the dialysis method. Dialysis using a semipermeable membrane will prevent the macromolecules of bentonite from passing into the amine solution, but it will allow the free passage of amine molecules. In this manner the amount of amine actually present in the solution in the vicinity of the bentonite could be compared to that calculated by the assay equation. The per cent deviation of amine found in the solution containing bentonite to that found in the solution in equilibrium with the former, but containing no bentonite, was $\pm 4.55\%$.

The reliability and accuracy of this assay was concluded to be adequate for the purpose of this study.

Determination of the Effect of Particle Size of Bentonite.—The particle size of resin exchangers does have an effect on the rate of exchange. The bentonite particle hydrates rapidly and seems to retain its original size. The hydrated particle did not disperse upon agitation; therefore, a study was performed to determine the effect of particle size. The bentonite was classified into six mesh sizes; those particles that did not pass through a 20-, 40-, 60-, 80-, and 100-mesh sieve, and those that passed through a 120-mesh sieve. The exchange rates were evaluated in the usual manner. The amount of amine exchanged was the same for all the particle sizes as the results indicate. See Table II. The penetration of the cation to the exchange site is rapid with acid activated bentonite.

It has been reported by other workers that the rate of exchange is rapid (1, 2, 5). The period of contact was selected as fifteen minutes since it was the most convenient and it would insure equilibrium of exchange and adsorption.

TABLE II.—EFFECT OF PARTICLE SIZE

Mesh	F[a]	X[b]	S. E.[c]	pH
20	0.4772	0.5230	0.0069	3.44
40	0.4925	0.5125	0.0071	3.45
60	0.4731	0.5307	0.0125	3.46
80	0.4558	0.5301	0.0147	3.45
100	0.4813	0.5186	0.0040	3.46
120	0.4813	0.5207	0.0076	3.45

[a] F, Equilibrium concentration, meq./100 ml. [b] X, Amount exchanged, meq./Gm. bentonite. [c] S. E., Standard error, ± values.

Determination of Exchange Isotherms.—The amount of amine exchanged per gram of bentonite was determined at various concentrations for each amine. The equilibrium concentration, amount exchanged per gram of bentonite, the standard error from the average amount exchanged, and the pH of the equilibrium solutions are listed for each amine. The pH of the solutions was determined with a Beckman model G pH meter. It is evident from the results that alkylation of the amino group and the position of the hydroxyl group affect the exchange rate. See Tables III–V.

Effect of pH on the Exchange Rate.—The effect of pH has been studied by previous investigators (1–3), but those studies were concerned with three different cations competing for an exchange site. The bentonite had been saturated with a dye, and organic cations plus hydrogen or ammonium ions were placed in aqueous contact with the dye-saturated bentonite. In this study the bentonite is hydrogen saturated and the organic cation is com-

TABLE III.—EXCHANGE ISOTHERM VALUES, PHENOLIC SUBSTITUTION SERIES

C[a]	F[b]	X[c]	S. E.[d]	pH
Hydroxyamphetamine HBr				
5.00	4.3117	0.6859	0.0608	3.20
4.00	3.3323	0.6626	0.0552	3.29
3.00	2.4402	0.5620	0.0471	3.28
2.00	1.5349	0.4728	0.0332	3.31
1.50	1.1207	0.4170	0.0075	3.34
1.00	0.6185	0.3772	0.0035	3.36
0.80	0.4440	0.3525	0.0015	3.41
0.70	0.3564	0.3420	0.0092	3.44
0.60	0.2868	0.3109	0.0076	3.51
0.56	0.2518	0.3065	0.0071	3.50
0.42	0.1596	0.2636	0.0101	3.55
0.40	0.1562	0.2429	0.0019	3.57
0.20	0.0691	0.1302	0.0020	3.92
0.10	0.0218	0.0782	0.0096	4.42
Phenylephrine HCl				
5.00	4.3375	0.6723	0.0460	3.29
4.00	3.3993	0.6074	0.0488	3.29
3.00	2.5086	0.4978	0.0590	3.29
2.00	1.5883	0.4137	0.0169	3.29
1.80	1.4247	0.3725	0.0101	3.29
1.60	1.2419	0.3545	0.0083	3.28
1.40	1.0672	0.3315	0.0096	3.31
1.00	0.7286	0.2766	0.0181	3.39
0.80	0.5803	0.2233	0.0101	3.47
0.60	0.4150	0.1865	0.0020	3.53
0.40	0.2649	0.1324	0.0106	3.61
0.20	0.1242	0.0744	0.0140	3.97
0.10	0.0602	0.0328	0.0024	4.25

[a] C, Initial concentration, meq./100 ml. [b] F, Equilibrium concentration, meq./100 ml. [c] X, Amount exchanged, meq./Gm. bentonite. [d] S. E., Standard error, ± values.

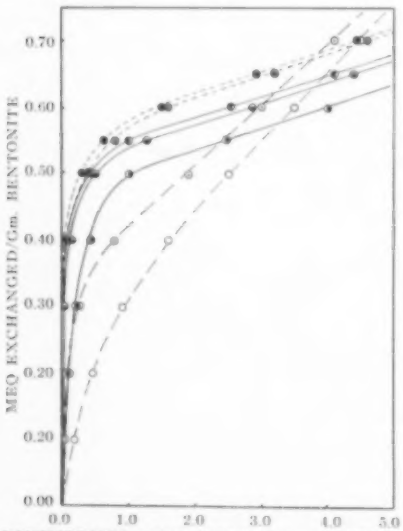

Fig. 3.—Exchange isotherms for amine series. ○ Phenylephrine HCl, ⊙ hydroxyamphetamine HBr, ◑ phenylpropanolamine HCl, ◒ ephedrine HCl, ⊗ amphetamine sulfate, ◐ 1-N-ethylephedrine HCl, ● methamphetamine HCl. – – – – – – phenolic hydroxyl, ———— primary, secondary, tertiary amine series, - - - - primary, secondary amines with no hydroxyl substitution.

TABLE IV.—EXCHANGE ISOTHERM VALUES, PRIMARY SECONDARY, TERTIARY AMINE SERIES

C[a]	F[b]	X[c]	S. E.[d]	pH
Phenylpropanolamine HCl				
5.00	4.3795	0.6225	0.0087	3.36
4.00	3.4275	0.5740	0.0360	3.34
3.00	2.4712	0.5367	0.0213	3.32
2.00	1.3800	0.5199	0.0347	3.36
1.50	1.0301	0.5010	0.0201	3.36
1.00	0.5520	0.4437	0.0054	3.37
0.80	0.4091	0.3904	0.0027	3.38
0.60	0.2658	0.3323	0.0047	3.39
0.48	0.1751	0.3051	0.0102	3.39
0.40	0.1498	0.2493	0.0011	3.40
0.36	0.1028	0.2574	0.0012	3.41
0.20	0.0471	0.1519	0.0134	3.62
0.10	0.0110	0.0858	0.0039	3.87
Ephedrine HCl				
5.00	4.3512	0.6466	0.0226	3.38
4.00	3.3838	0.6135	0.0195	3.38
3.00	2.4282	0.5773	0.0317	3.38
2.00	1.4501	0.5506	0.0225	3.39
1.50	0.9910	0.5331	0.0278	3.39
1.00	0.5076	0.4997	0.0288	3.37
0.80	0.3543	0.4679	0.0143	3.38
0.60	0.1899	0.4186	0.0093	3.38
0.50	0.1169	0.3801	0.0099	3.39
0.40	0.0619	0.3361	0.0034	3.36
0.30	0.0301	0.2715	0.0086	3.40
0.20	0.0138	0.1847	0.0029	3.50
1-N-Ethylephedrine HCl				
5.00	4.3412	0.6558	0.0397	3.21
4.00	3.3709	0.6297	0.0424	3.21
3.00	2.3812	0.6166	0.0314	3.21
2.00	1.4421	0.5561	0.0500	3.22
1.50	0.9611	0.5441	0.0278	3.22
1.00	0.4673	0.5035	0.0332	3.22
0.80	0.3231	0.4781	0.0026	3.24
0.60	0.1618	0.4371	0.0027	3.26
0.40	0.0282	0.3705	0.0030	3.32
0.32	0.0102	0.3090	0.0113	3.32
0.24	0.0070	0.2330	0.0202	3.41
0.20	0.0031	0.1934	0.0089	3.48

[a] C, Initial concentration, meq./100 ml. [b] F, Equilibrium Concentration, meq./100 ml. [c] X, Amount exchanged, meq./Gm. bentonite. [d] S. E., Standard error, ± values.

peting with hydrogen ions alone for the exchange site. Also, the nature and extent of adsorption might be more clearly defined by comparison of acid and basic exchange values. The pH of the initial contact solution was adjusted to the desired level with 0.121 *N* hydrochloric acid or 10% ammonia solution in all the experiments in this study. See Tables VI–VIII.

A study performed to determine the effect of the ammonium ion on exchange rates indicated that its presence in the concentrations used to establish the pH level affected the exchange rate to a negligible degree. The usual procedure for determining exchange was followed.

The spectra of the amines are changed in solutions of basic pH; therefore, the equilibrium solutions were adjusted to pH 3.5 to 4.0 before being assayed. The amines with phenolic hydroxyl groups are not stable in basic pH media, and the results of the experiments run at pH 10 for these amines were erratic. Therefore, only the results for the alkylation of amino group and those with no hydroxyl substitution are listed at pH levels of 10 and 2.

TABLE V.—EXCHANGE ISOTHERM VALUES, AMINE SERIES WITH NO HYDROXYL SUBSTITUTION

C[a]	F[b]	X[c]	S. E.[d]	pH
Amphetamine Sulfate				
5.00	4.2958	0.6827	0.0362	3.60
4.00	3.3686	0.6339	0.0378	3.59
3.00	2.3810	0.6189	0.0512	3.59
2.00	1.4030	0.5919	0.0502	3.59
1.50	0.9700	0.5520	0.0411	3.63
1.00	0.4957	0.5061	0.0359	3.65
0.80	0.3471	0.4471	0.0057	3.66
0.60	0.1882	0.4117	0.0022	3.66
0.50	0.1161	0.3796	0.0065	3.67
0.40	0.0545	0.3419	0.0015	3.67
0.30	0.0347	0.2704	0.0076	3.66
0.20	0.0168	0.1823	0.0010	3.80
Methamphetamine HCl				
5.00	4.3109	0.6897	0.0490	3.16
4.00	3.3463	0.6576	0.0600	3.19
3.00	2.3560	0.6265	0.0510	3.19
2.00	1.3996	0.5778	0.0450	3.28
1.50	0.9450	0.5660	0.0226	3.26
1.00	0.4730	0.5319	0.0256	3.26
0.80	0.2983	0.4988	0.0054	3.26
0.60	0.1440	0.4527	0.0032	3.27
0.40	0.0179	0.3773	0.0030	3.28
0.32	0.0094	0.3136	0.0096	3.28
0.24	0.0031	0.2402	0.0083	3.29
0.20	0.0031	0.1978	0.0017	3.47

[a] C, Initial concentration, meq./100 ml. [b] F, Equilibrium concentration, meq./100 ml. [c] X, Amount exchanged, meq./Gm. bentonite. [d] S. E., Standard error, ± values.

TABLE VI.—AMOUNT OF AMINE EXCHANGED AT pH = CONSTANT

Group	Amine	X[a]	pH
1	Phenylephrine HCl	0.3545	3.28
	Hydroxyamphetamine HBr	0.3409	3.50
	Phenylpropanolamine HCl	0.3323	3.39
2	Ephedrine HCl	0.3261	3.36
	1-N-Ethylephedrine HCl	0.3302	3.26
3	Amphetamine sulfate	0.3054	3.67
	Methamphetamine HCl	0.3136	3.28
	NaCl	0.3135	3.62
	KCl	0.3247	3.54

[a] X, Amount exchanged, meq./Gm. bentonite.

Effect of Ionic Strength and the Nature of the Electrolyte.—It has been reported in many studies that the nature of the electrolyte cation and the ionic strength of the contact solution have an effect on exchange reactions. A study was made to determine the quantitative changes produced by varying the added cation and ionic strength. The ionic strength of the initial contact solution was adjusted to values of 0.02, 0.05, 0.10, and 0.20 for all the amine solutions. The ionic strengths were adjusted using sodium chloride and potassium chloride. The added salts increased the extinction of the amines slightly; therefore, the blank solution was prepared containing the added salt. The effect was generally the same for all the amines; therefore, only the results for phenylpropanolamine HCl, amphetamine sulfate, and 1-N-ethylephedrine HCl are presented in Table IX.

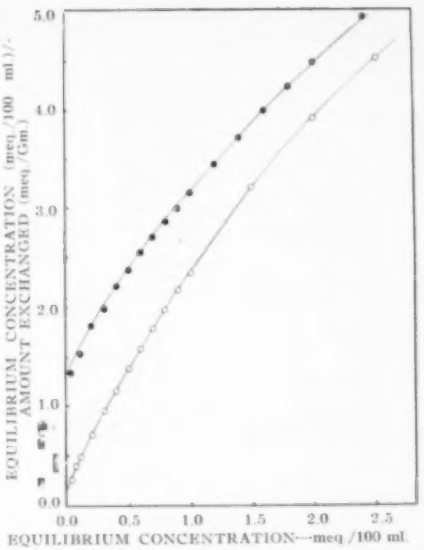

Fig. 4.—Langmuir plot of phenylephrine HCl and hydroxyamphetamine HBr. ● Phenylephrine HCl, ○ hydroxyamphetamine HBr.

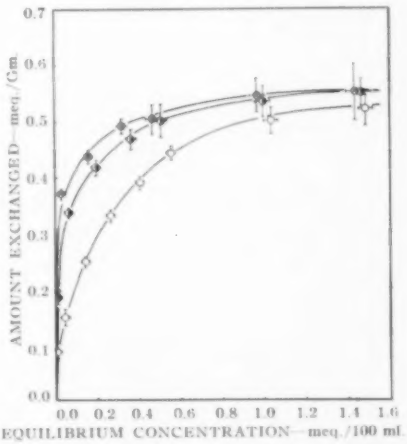

Fig. 5.—Exchange isotherms of primary secondary, tertiary amine series. ○ Phenylpropanolamine HCl, ◑ ephedrine HCl, ⊗ 1-N-ethylephedrine HCl. Vertical and horizontal bars are the 95% confidence intervals.

DISCUSSION

The sorption reaction, both exchange and adsorption occur, reaches equilibrium within fifteen minutes with constant agitation. It was observed during the experiments determining the effect of particle size that there was little change in the size of the particles. Although acid activation decreases hydration of bentonite, the amount of hydration is sufficient to maintain the hydrated particle size constant during agitation, and it also allows the

TABLE VII.—EFFECT OF pH ON EXCHANGE, AMINES WITH NO HYDROXYL SUBSTITUTION

C[a]	pH = 10 F[b]	pH = 10 X[c]	pH = 10 S.E.[d]	pH = 2 F	pH = 2 X	pH = 2 S.E.
			Amphetamine Sulfate			
2.0	1.3433	0.6596	0.0232	1.4331	0.5410	0.0351
1.5	0.8936	0.6106	0.0162	0.9906	0.5078	0.0167
1.0	0.4539	0.5451	0.0177	0.5171	0.4784	0.0232
0.8	0.3121	0.4906	0.0101	0.3738	0.4315	0.0076
0.6	0.1752	0.4345	0.0042	0.1952	0.3290	0.0032
0.4	0.0288	0.3755	0.0078	0.0753	0.3290	0.0041
0.2	0.0005	0.1994	0.0072	0.0331	0.1680	0.0018
			Methamphetamine HCl			
2.0	1.3037	0.7015	0.0323	1.4785	0.5207	0.0217
1.5	0.8276	0.6810	0.0275	1.0260	0.4897	0.0266
1.0	0.3459	0.6511	0.0178	0.5305	0.4708	0.0117
0.8	0.1709	0.6264	0.0112	0.3342	0.4647	0.0087
0.6	0.0602	0.5355	0.0166	0.1528	0.4461	0.0101
0.4	0.0090	0.3920	0.0086	0.0191	0.3795	0.0075
0.2	0.0005	0.2000	0.0101	0.0064	0.1919	0.0070

[a] C, Initial concentration, meq./100 ml. [b] F, Equilibrium concentration, meq./100 ml. [c] X, Amount exchanged, meq./Gm. bentonite. [d] S. E., Standard error, ± values.

TABLE VIII.—EFFECT OF pH ON EXCHANGE, PRIMARY, SECONDARY, TERTIARY AMINE SERIES

C[a]	pH = 10 F[b]	pH = 10 X[c]	pH = 10 S.E.[d]	pH = 2 F	pH = 2 X	pH = 2 S.E.
			Phenylpropanolamine HCl			
2.0	1.4116	0.5783	0.0276	1.5377	0.4618	0.0095
1.5	0.9759	0.5247	0.0223	1.0692	0.4392	0.0085
1.0	0.5591	0.4676	0.0123	0.5845	0.4091	0.0073
0.8	0.3903	0.4342	0.0134	0.4335	0.3700	0.0043
0.6	0.2496	0.3676	0.0101	0.2807	0.3213	0.0067
0.4	0.1313	0.2767	0.0080	0.1603	0.2416	0.0034
0.2	0.0389	0.1622	0.0070	0.0516	0.1486	0.0046
			Ephedrine HCl			
2.0	1.3906	0.6187	0.0288	1.5006	0.4954	0.0112
1.5	0.9107	0.5961	0.0307	1.0212	0.4802	0.0168
1.0	0.4426	0.5641	0.0187	0.5372	0.4487	0.0095
0.8	0.2637	0.5407	0.0107	0.3770	0.4256	0.0076
0.6	0.1382	0.4626	0.0125	0.2101	0.3976	0.0046
0.4	0.0228	0.3769	0.0073	0.0809	0.3212	0.0068
0.2	0.0036	0.1977	0.0088	0.0290	0.1715	0.0055
			1-N-Ethylephedrine HCl			
2.0	1.3507	0.6512	0.0289	1.4757	0.5204	0.0076
1.5	0.8702	0.6317	0.0299	0.9912	0.5102	0.0104
1.0	0.3927	0.6072	0.0171	0.5160	0.4903	0.0049
0.8	0.2462	0.5732	0.0161	0.3371	0.4650	0.0067
0.6	0.1006	0.5012	0.0101	0.1761	0.4280	0.0055
0.4	0.0139	0.3899	0.0076	0.0487	0.3517	0.0041
0.2	0.0013	0.1977	0.0083	0.0082	0.1888	0.0062

[a] C, Initial concentration, meq./100 ml. [b] F, Equilibrium concentration, meq./100 ml. [c] X, Amount exchanged, meq./Gm. bentonite. [d] S. E., Standard error, ± values.

rapid penetration of the organic cation to the exchange site. The nature and exact position of the exchange site on bentonite is not known (7), but it is postulated that the exchangeable cations are associated with the lattice to neutralize unequal charges, which are produced by substitution of ions of lower valency in the lattice.

The exchange isotherms for the amine series are represented in Fig. 3. For clarity, the curves are an ideal representation of the experimental results. The amines can be separated into three groups: (*a*) phenolic hydroxyl substitution; (*b*) primary, secondary, tertiary amine series with β-hydroxyl substitution; (*c*) amines with no hydroxyl substitution.

The amount of amine exchanged, when constant pH is attained, is listed in Table VII. Comparison of these values with those of the inorganic cations indicates simultaneous occurrence of exchange and adsorption.

The amount of amine exchanged at the constant pH level decreases as the hydroxyl substitution of the amine decreases. The isotherms for hydroxyamphetamine HBr and phenylephrine HCl are very similar.

The presence of the phenolic hydroxyl group appears to decrease the amount of exchange and substance as compared to the amines with no such substitution. Since the amount removed from solution is much greater, by comparison, for these two compounds, the phenolic hydroxyl group appears to enhance adsorption. If the results are represented

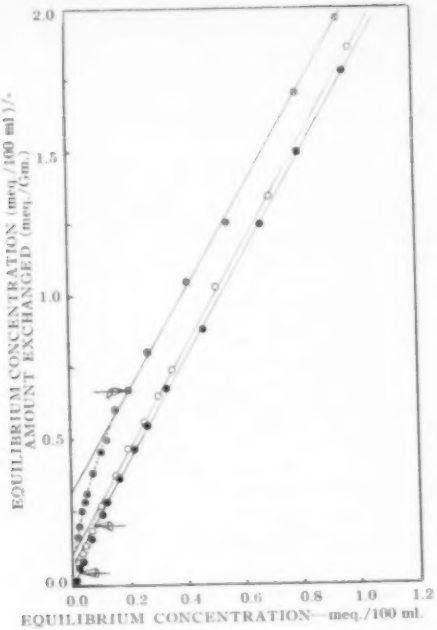

Fig. 6.—Langmuir plot of primary, secondary, tertiary amine series. ⊗ Phenylpropanolamine HCl, ○ ephedrine HCl, ● 1-N-ethylephedrine HCl, ← constant pH.

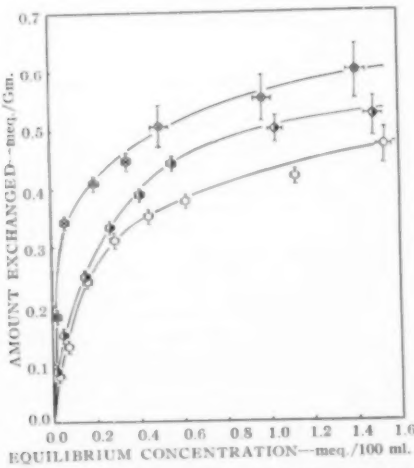

Fig. 7.—Exchange isotherms, hydroxyl position effect. ○ Hydroxyamphetamine HBr, ◑ phenylpropanolamine HCl, ⊗ amphetamine sulfate. Vertical and horizontal bars are the 95% confidence intervals.

as a Langmuir adsorption isotherm (Fig. 4), these two compounds show deviation at low concentration and at high concentration. The deviation at low concentration is due to the exchange reaction, while the deviation at high concentration is indicative of

TABLE IX.—EFFECT OF IONIC STRENGTH

C[a]	Salt	u[b] = 0.02 X[c]	u = 0.05 X	u = 0.10 X	u = 0.20 X
		Phenylpropanolamine HCl			
0.2	NaCl	0.1501	0.1482	0.1452	0.1425
	KCl	0.1497	0.1481	0.1447	0.1422
0.5	NaCl	0.3027	0.2992	0.2962	0.2902
	KCl	0.3007	0.2967	0.2927	0.2862
1.0	NaCl	0.4262	0.4212	0.4072	0.3892
	KCl	0.4302	0.4172	0.4056	0.3865
		1-N-Ethylephedrine HCl			
0.2	NaCl	0.1924	0.1899	0.1862	0.1812
	KCl	0.1920	0.1888	0.1870	0.1826
0.5	NaCl	0.4007	0.3970	0.3901	0.3872
	KCl	0.3897	0.3862	0.3807	0.3701
1.0	NaCl	0.4922	0.4759	0.4583	0.4171
	KCl	0.4875	0.4702	0.4607	0.4186
		Amphetamine Sulfate			
0.2	NaCl	0.1800	0.1780	0.1740	0.1680
	KCl	0.1830	0.1720	0.1720	0.1610
0.5	NaCl	0.4070	0.4040	0.3960	0.3830
	KCl	0.3790	0.3750	0.3690	0.3550
1.0	NaCl	0.5029	0.4850	0.4640	0.4140
	KCl	0.4720	0.4560	0.4420	0.3910

[a] C, Initial concentration, meq./100 ml. [b] u, Ionic strength. [c] X, Amount exchanged, meq./Gm. bentonite.

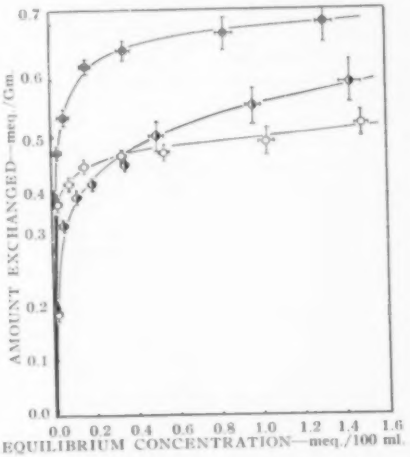

Fig. 8.—Effect of pH on the exchange isotherm of methamphetamine HCl. ○ pH 2, ◑ pH 6, ⊗ pH 10. Vertical and horizontal bars are the 95% confidence intervals.

multilayer adsorption, which may occur by means of hydrogen bonding.

In the second group the amount exchanged at the constant pH level is essentially the same. The isotherms for this group are presented in Fig. 5. The extent of exchange increases with alkylation of the amino group. The isotherms indicate parallel rates of adsorption after reaching the constant pH point. The results of this group were subjected to the Langmuir analysis (Fig. 6). The Langmuir plots indicate that adsorption is the controlling reaction after a constant pH level is reached. The deviations from the Langmuir plot at low concentration are

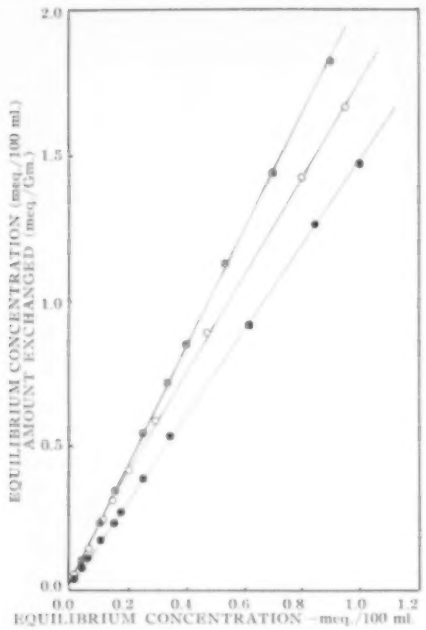

Fig. 9.—Langmuir plot of pH effect on the exchange isotherm of methamphetamine HCl. ⊗ pH 2, ○ pH 6, ● pH 10.

due to the exchange reaction predominating. The extent of exchange is greater than that of adsorption.

The results of the third group indicate that removal of the hydroxyl from the beta position increases the extent or exchange and that the secondary amine has the greater. A comparison of the isotherms for a member of each group is presented in Fig. 7. Langmuir plots of the data of this group are very similar to those of the second group, indicating the occurrence of exchange and adsorption.

Since the alkylation of the amino group increases the extent of exchange, a comparison of the pKb values of the amines was made (Table X). It is apparent that the amount of amine removed from solution increases with basicity or decreasing pKb values.

Table X.—Amine Series pKb Values

Amine	X[a]	X[b]	pKb[c]
Methamphetamine HCl	0.5320	0.3850	4.13
Amphetamine sulfate	0.5140	0.3500	4.23
l-N-Ethylephedrine HCl	0.5120	0.3700	[d]
Ephedrine HCl	0.5020	0.3440	4.42
Phenylpropanolamine HCl	0.4480	0.2600	4.56
Hydroxyamphetamine HBr	0.3830	0.2480	4.69
Phenylephrine HCl	0.2680	0.1330	5.14

[a] X, Amount exchanged, meq./Gm. when initial concentration is 1.0 meq./100 ml. [b] X, Amount exchanged, meq./Gm. when initial concentration is 0.4 meq./100 ml. [c] pKb values source, ref. (8). [d] pKb value not obtainable.

It is evident from the results shown in Tables VIII and IX that the extent of exchange increased with an increase in pH. The ratio of protonated amine to amine base decreases as pH increases; therefore, the amount exchanged should decrease. In effect, the amount exchanged increases to a value greater than that at the natural pH of the amine salt solution (Fig. 8). The controlling reaction at pH 10 is adsorption; whereas, the controlling reaction at pH 2 appears to be ion exchange. Figure 9 is a Langmuir plot of the results for methamphetamine HCl at pH 2, 6, and 10. The results at pH 2 and 10 give straight lines with slopes of 2.000 and 1.471, respectively. The straight line portion of the pH 6 (natural pH of hydrochloride salt) plot has a slope of 1.666, which indicates that adsorption is predominating. The pH 6 results follow a straight line until they intersect the pH 2 curve, at which point the pH 6 curve follows the pH 2 plot. If it is assumed that at pH 10 adsorption is the controlling reaction and ion exchange is the controlling reaction at pH 2, then, a quantitative relation of these two effects can be determined from this type of plot. In a subsequent article this relation between adsorption and exchange will be discussed.

In all concentration ranges, increasing the ionic strength decreases the extent of exchange, which would be expected. A comparison of the amount exchanged as ionic strength increases shows that the per cent decrease is nearly the same for all amines studied. In the experiments in which the amine concentration is low, a tenfold increase in the number of inorganic cations ($u = 0.02$ to $u = 0.20$) reduces the amount of amine exchanged 5% in the case of phenylpropanolamine HCl. The per cent decreases in amount exchanged as ionic strength increases is not much greater at higher concentrations.

It appears that the effects of ionic strength is not as significant in organic exchange reactions. This is rational in view of the ionic and molecular forces present in exchange of organic ions. The combined molecular and ionic forces of the organic cation are great enough to overcome the stronger ionic force of the inorganic cation.

The effect of the electrolyte cation follows the usual pattern associated with exchange reactions (5). The hydrated ionic diameter of the ion is related to the replacing power or exchange affinity; therefore, the potassium ions, with a larger hydrated ionic diameter, have a greater effect on the organic ion exchange reaction than the sodium ions.

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Triterpenoids XI. New Triterpenoid Sapogenins from the Fruits of *Barringtonia acutangula*

By A. K. BARUA†, P. C. MAITI, and SACHINDRA K. CHAKRABORTI

Isolation of three new triterpenoid sapogenols called barringtogenol B, $C_{30}H_{50}O_6$, barringtogenol C, $C_{30}H_{50}O_5$, and barringtogenol D, $C_{30}H_{48-50}O_4$, is reported from the fruits of *Barringtonia acutangula* Gaertn. Two triterpenoid acid sapogenins have also been isolated from the same source through their methyl esters, $C_{31}H_{50}O_4$ and $C_{32}H_{50}O_6$. The latter has been identified as methyl barringtogenate.

BARRINGTONIA ACUTANGULA Gaertn., commonly known in Bengal as hijal, is widely distributed in India. The seeds of the plant were found to contain saponins by Lahiri and Ghosh (1). Kinel and Gedeon (2) reported the isolation of three sapogenols melting at 173–175°, 270–275°, and 310–312°, respectively, from the root, bark, and seeds of *B. acutangula* Gaertn., and *B. racemosa* Roxb. From the fruit kernels of the latter, Pillai and Anantaraman (3) isolated two sapogenins, barringtogenol, $C_{30}H_{50}O_4$, m. p. 290–291°, and barringtogenic acid, $C_{30}H_{46}O_6$, m. p. 332–334°. They established the structure of barringtogenol and barringtogenic acid as 2,3,23,-28-tetrahydroxyolean-12-ene and 2,3-dihydroxy-olean-12-ene-23,28-dioic acid, respectively.

In our preliminary communication (4) the isolation of four triterpenoid sapogenins from *B. acutangula* was reported and the present paper describes the isolation of another new triterpenoid sapogenol and furnishes additional data on the sapogenins previously reported.

DISCUSSION

The ethanolic extract of dry and defatted fruits of *B. acutangula* gave a large amount of saponins. Acid hydrolysis of the crude saponin furnished a good quantity of crude sapogenin which was percolated with sufficient ether in the cold, and the ether-soluble sapogenin was separated into acid and neutral fractions called A and B, respectively. The ether-insoluble sapogenin (fraction C) was treated separately. Fraction A, on treatment with diazomethane, yielded two methyl esters, $C_{32}H_{50}O_6$ (I), m. p. 253–254°; $[\alpha]_D^{26}$ + 65° (methanol) and $C_{31}H_{50}O_4$ (II), m. p. 301–303°. The yield of the latter being poor, further work on it was not feasible. The ester (I) responded to the Liebermann-Burchard test and gave a pale yellow coloration with tetranitromethane. It formed a diacetate, m. p. 239–240°, $[\alpha]_D^{31}$ + 31.5° (methanol). The physical constants of the methyl ester (I) and its diacetate were found to be in close agreement with those of methyl barringtogenate and its diacetate. The melting point of the mixture of methyl ester (I) with an authentic sample of methyl barringtogenate showed no depression. The methyl ester (I), on lithium aluminum hydride reduction, furnished barringtogenol, m. p. 288°, undepressed when mixed with an authentic sample of the latter prepared from arjunolic acid (5).

Fraction B was resolved by chromatography over alumina into two crystalline colorless compounds, $C_{30}H_{50}O_6$ (III), m. p. 249°, $[\alpha]_D^{30}$ + 6° (chloroform); and $C_{30}H_{50}O_5$ (IV), m. p. 315–320° (decompn.), $[\alpha]_D^{28}$ + 33.8° (dioxane). Both the compounds responded to the Liebermann-Burchard test and gave pale yellow coloration with tetranitromethane. The yield of the product (III) was insufficient for further study. The compound (IV) formed a pentaacetate, $C_{40}H_{60}O_{10}$, m. p. 222–224°, $[\alpha]_D^{30}$ + 18° (ethanol); and a pentabenzoate, $C_{65}H_{70}O_{10}$, m. p. 315–317°, $[\alpha]_D^{29}$ + 30.9° (dioxane), both of which on hydrolysis gave the compound (IV).

Compounds (III) and (IV), were new triterpene compounds, and were named acutagenol A and acutagenol B, respectively (4). But now we feel that these compounds should be called barringtogenol B and barringtogenol C, respectively, in conformity with barringtogenol and barringtogenic acid reported from *Barringtonia racemosa* (3).

Barringtogenol C (IV) contains a hindered double bond, as its pentaacetate and pentabenzoate consumed only 0.7 mole of perbenzoic acid in fifteen days. The double bond could not be catalytically hydrogenated. It consumed one mole of periodic acid indicating the presence of a 1:2-diol system in the molecule.

The ether-insoluble crude sapogenin mixture (fraction C) was exhaustively extracted with ethyl acetate and the residue obtained from the ethyl acetate solution was benzoylated. Chromatographic resolution of the product gave a glassy solid (fraction D), m. p. 145–160°, and a crystalline fraction (E). The glassy solid did not crystallize and could not be purified further by chromatography. It was acetylated after hydrolysis and the resulting product was purified by chromatography over alumina, when a crystalline triacetate, $C_{36}H_{54-56}O_7$, m. p. 233–234°, $[\alpha]_D^{32}$ + 74° (chloroform) was obtained. It furnished, on hydrolysis, an alcohol, $C_{30}H_{48-50}O_4$ (V), m. p. 305–310°. On benzoylation, this yielded a glassy product but acetylation gave

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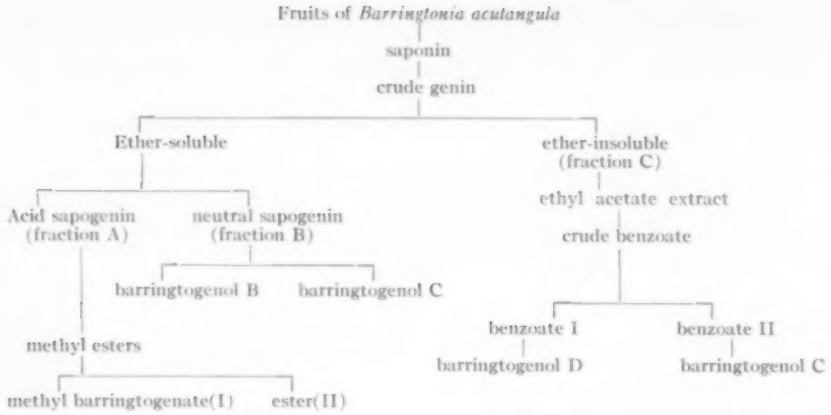

the above triacetate. The alcohol (V) appears to be a new triterpene alcohol and is named barringtogenol D. The crystalline material obtained in fraction E, m. p. 315–317°, $[\alpha]_D^{25}$ + 30.9° (dioxane), was found to be identical with barringtogenol C pentabenzoate by mixed melting point. This, on hydrolysis, gave barringtogenol C.

The schematic diagram of the process for isolating the different fractions is given.

Further work on the constitution of barringtogenol C and barringtogenol D is under progress.

EXPERIMENTAL

Melting points were determined in a bisulfate bath and are uncorrected. Unless otherwise mentioned the alumina used in the chromatography refers to Brockmann's alumina, deactivated by shaking in benzene with 5% of 10% acetic acid. The petroleum ether used boiled at 60–80°. The analytical samples were dried in vacuum over phosphorus pentoxide at 140°.

Isolation of Sapogenins.—The dry powdered fruits (2.5 Kg.) were defatted with petroleum ether in a Soxhlet apparatus for thirty hours. The material was then exhaustively extracted with boiling ethanol (95%) for sixty hours. The ethanolic extract was concentrated to a small bulk (300 ml.) and a large amount of saponin was precipitated as a gummy mass on dilution with ether. It was repeatedly washed with ether, and the ether-insoluble brown, gummy substance thus obtained, responded to the characteristic tests for saponin. The crude saponin was then taken up in 300 ml. of water and 700 ml. of 95% ethanol and was hydrolyzed under reflux for four hours after addition of 200 ml. of hydrochloric acid. The product was poured into a large basin and the alcohol was removed on a water bath, keeping the volume constant by gradual addition of water. The dark-colored sapogenin thus precipitated was filtered, washed, and dried; yield, 95 Gm.

The above crude sapogenin was soaked in 500 ml. of ether in the cold for twenty-four hours and the solvent was decanted. The process was repeated three times and the combined ether extract was treated separately. The ether-insoluble sapogenin (fraction C) was subsequently extracted with 600 ml. of ethyl acetate in a Soxhlet apparatus for forty-eight hours. The extract, on concentration, yielded 22 Gm. of a dark solid.

Treatment of the Ether-Soluble Sapogenin.—The ethereal extract of the crude sapogenin was shaken with 500 ml. of 5% aqueous caustic soda. When the sodium salt of the acid sapogenin precipitated, it was filtered and acidified with hydrochloric acid and free acid sapogenins were obtained as a white solid; yield, 1.1 Gm. (fraction A). The ether layer was separated from the filtrate, washed with water, and dried over anhydrous sodium sulfate. The aqueous layer, on acidification, yielded a small quantity of acidic material.

On removing the solvent from the ethereal solution, 3.5 Gm. of a viscous, yellow, neutral substance was obtained (fraction B).

Treatment of Fraction A.—*Isolation of Methyl Barringtogenate.*—The crude acid (fraction A) was esterified with diazomethane in ether solution and the methyl ester thus obtained was taken up in 15 ml. of benzene and adsorbed on a column of acid-washed alumina (60 Gm.). Elution with 500 ml. of benzene-ether mixture (1:1) gave a fraction which, on crystallization from methanol, melted at 253–254° (I), $[\alpha]_D^{25}$ + 65° (methanol); yield, 1.0 Gm.

Anal.—Calcd. for $C_{32}H_{50}O_6$: C, 72.40; H, 9.51. Found: C, 72.0, 72.2; H, 9.5, 9.5.

It did not depress the melting point of an authentic sample of methyl barringtogenate. Further elution of the column with 900 ml. of ethyl acetate furnished a colorless, crystalline compound, m. p. 301–303° (II); yield, 50 mg.

Anal.—Calcd. for $C_{31}H_{50}O_4$: C, 76.49; H, 10.35. Found: C, 76.20; H, 10.33.

Lithium Aluminum Hydride Reduction of Methyl Barringtogenate.—Methyl barringtogenate (0.5 Gm.) in 100 ml. of dry ether with 1 Gm. of lithium aluminum hydride was refluxed for three hours and left overnight. The excess reagent was decomposed by cautious addition of cold water and 50 ml. of dilute sulfuric acid was added to decompose the metal complex. The ethereal solution was separated, washed with water, dried, and evaporated to dryness. The residue (300 mg.) was crystallized from acetone; m. p. 288° (undepressed with lithium aluminum hydride reduction product of methyl arjunolate).

Anal.—Calcd. for $C_{30}H_{46}O_4$: C, 75.90; H, 10.62. Found: C, 75.8; H, 10.5.

Preparation of Barringtogenol from Arjunolic Acid.—The methyl ester of arjunolic acid (150 mg.) was reduced with lithium aluminium hydride as described for methyl barringtogenate. The product melted at 278–280° and, on repeated crystallization from acetone, gave fine needles, m. p. 286–287°.

Anal.—Calcd. for $C_{30}H_{50}O_4$: C, 75.90; H, 10.62. Found: C, 75.72; H, 10.70.

Preparation of Diacetate of Methyl Barringtogenate.—Methyl barringtogenate (100 mg.) was heated on a water bath with 5 ml. of pyridine and 2 ml. of acetic anhydride for one hour. On working up in the usual way a product was obtained (m. p. 235–238°) which crystallized in fine needles from methanol; m. p. 239–240°, $[\alpha]_D^{31}$ +31.5° (methanol).

Anal.—Calcd. for $C_{34}H_{54}O_6$: C, 70.31; H, 8.85. Found: C, 69.91; H, 8.88.

Treatment of Fraction B.—*Isolation of Barringtogenol B and C.*—The neutral fraction B (3.5 Gm.) was dissolved in 20 ml. of benzene and adsorbed on a column of alumina (200 Gm.). Elution with 500 ml. of benzene gave a viscous liquid which could not be crystallized. Further elution of the column with 750 ml. of benzene-ether (1:2) yielded an impure product, m. p. 120–185°. A compound (III), barringtogenol B, m. p. 249°, could be obtained from the latter fraction after repeated crystallization from benzene and methanol; yield, 150 mg.

Anal.—Calcd. for $C_{30}H_{50}O_6$: C, 71.11; H, 9.94. Found: C, 71.38; H, 10.25.

Elution of the same column with 600 ml. of ethyl acetate gave a fraction which, on crystallization from dioxane-benzene and dioxane-alcohol, furnished a crystalline compound(IV), barringtogenol C, m. p. 315–320° (decompn.), $[\alpha]_D^{28}$ + 33.8° (dioxane); yield, 300 mg.

Anal.—Calcd. for $C_{30}H_{50}O_6$: C, 73.42; H, 10.27. Found: C, 73.1; H, 10.33.

Preparation of the Pentabenzoate of Barringtogenol C.—Barringtogenol C (100 mg.) was dissolved in 5 ml. of pyridine and heated on a water bath with 0.5 ml. of benzoyl chloride for six hours. The product was poured into crushed ice and stirred well. The separated solid was filtered and washed thoroughly with boiling water until free from benzoic acid. The benzoate crystallized from chloroform-methanol as colorless needles, m. p. 315–317°, $[\alpha]_D^{28}$ + 30.9° (dioxane).

Anal.—Calcd. for $C_{65}H_{70}O_{10}$: C, 77.23; H, 6.98. Found: C, 77.25; H, 7.13.

Preparation of the Pentaacetate of Barringtogenol C.—Barringtogenol C (100 mg.) was heated on a water bath for three hours with 1 ml. of acetic anhydride and 5 ml. of pyridine, and the product was worked up in the usual way. Repeated crystallization of the product from aqueous ethanol (charcoal) afforded the crystalline acetate, m. p. 222–224°, $[\alpha]_D^{28}$ + 18° (ethanol).

Anal.—Calcd. for $C_{50}H_{60}O_{10}$: C, 68.54; H, 8.62. Found: C, 68.41; H, 8.70.

Further Treatment of the Fraction C.—*Isolation of Barringtogenol C.*—The dark brown solid (22 Gm.) obtained in the ethyl acetate extract of the crude sapogenin was dissolved in 200 ml. of dry pyridine and heated on the water bath with 25 ml. of benzoyl chloride for five hours. The mixture, while hot, was poured into crushed ice and left overnight. The solidified matter was filtered, washed repeatedly with boiling water, and dried. The crude benzoate was dissolved in 75 ml. of benzene and chromatographed over a column of alumina (600 Gm.). A brown, glassy material (5.0 Gm.), m. p. 135–150° (fraction D), was obtained from the 2 L. of petroleum ether eluate. Subsequent elution with 4 L. of petroleum ether-benzene mixture yielded 13 Gm. of a crystalline solid (fraction E), m. p. 305–310°. This was repeatedly crystallized from chloroform-methanol as colorless needles, m. p. 315–317°, $[\alpha]_D^{28}$ + 30.9° (dioxane).

Anal.—Calcd. for $C_{65}H_{70}O_{10}$: C, 77.23; H, 6.98. Found: C, 77.21; H, 7.16.

A mixed melting point determination with barringtogenol C pentabenzoate recorded no depression.

The above crystalline benzoate (5 mg.) was then hydrolyzed under reflux for four hours with 300 ml. of 10% ethanolic caustic potash and the hydrolyzed product was worked up in the usual way. It was found to be sparingly soluble in most of the organic solvents and was crystallized repeatedly from dioxane-ethanol and dioxane-benzene as colorless shining needles (2.6 Gm.), m. p. 315–320°, identical with barringtogenol C.

Anal.—Calcd. for $C_{30}H_{50}O_6$: C, 73.42; H 10.27. Found: C, 73.15; H, 10.23.

A sample of this compound on benzoylation gave a benzoate, m. p. 315–317°, undepressed when mixed with the original pentabenzoate of barringtogenol C.

Isolation of Barringtogenol D.—The crude glassy substance (fraction D) was rechromatographed over a column of alumina (150 Gm.) and a colorless glassy material, m. p. 146–160°, was obtained in the petroleum ether eluate. Purification of this substance by crystallization was not fruitful. It was hydrolyzed under reflux for four hours with 10% ethanolic caustic potash. The product on working up was found to be still glassy and did not crystallize well. It was then acetylated with 10 ml. of pyridine and 5 ml. of acetic anhydride in the usual way. The crystalline acetate thus obtained, on repeated crystallization from aqueous ethanol, was not pure and melted at 215–225°. Chromatography of this material in 10 ml. of petroleum ether-benzene mixture over alumina (100 Gm.) and elution with 300 ml. of petroleum ether-benzene (2:1) yielded 2 Gm. of a crystalline compound, m. p. 228–230°. It was crystallized from ethanol as constant melting colorless needles, m. p. 233–234°, $[\alpha]_D^{32}$ + 74° (chloroform).

Anal.—Calcd. for $C_{36}H_{54}O_7$: C, 72.20; H, 9.09; Calcd. for $C_{36}H_{56}O_7$: C, 71.96; H, 9.39. Found: C, 71.93; 72.27; H, 9.06, 8.93.

Acetyl value calculated for triacetate: 21.5%; di- and tetraacetate, 14.3 and 26.7%, respectively (on the basis of $C_{36}H_{56}O_7$). Found: 22.6%.

Hydrolysis of the above acetate under reflux with 150 ml. of 10% ethanolic caustic potash and crystallization of the hydrolyzed product from ethanol gave 1.2 Gm. of colorless flakes, m. p. 305–310°.

Anal.—Calcd. for $C_{30}H_{48}O_4$: C, 76.25; H, 10.23; Calcd. for $C_{30}H_{50}O_4$: C, 75.90; H, 10.61. Found: C, 76.32; H, 10.39.

Perbenzoic Acid Titration of Pentabenzoate and Pentaacetate of Barringtogenol C.—The penta-

benzoate and pentaacetate of barringtogenol C (90 mg. of each), dissolved separately in 5 ml. of chloroform, were treated with 5 ml. of 0.3 *N* perbenzoic acid in chloroform at 0°. Each of the compounds consumed about 0.7 mole of perbenzoic acid in fifteen days.

Periodic Acid Titration of Barringtogenol C.—Barringtogenol C (40 mg.) in 20 ml. of methanol was treated with 1 ml. of 0.5 *M* periodic acid and the excess periodic acid was titrated with standard arsenite solution after twenty-four hours. It consumed 1.1 mole of periodic acid.

Attempted Hydrogenation of the Pentaacetate of Barringtogenol C.—Pentaacetate of barringtogenol C (30 mg.) in glacial acetic acid was shaken in an atmosphere of hydrogen in presence of Adam's catalyst for ten hours. After working up in the usual way the original product was obtained.

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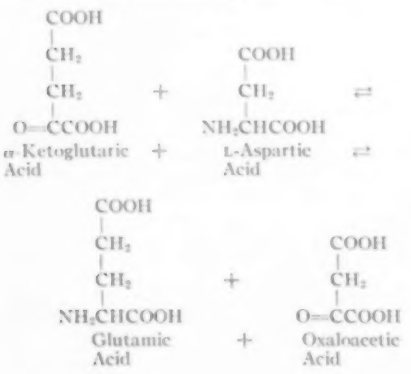
Comparison of Transaminase Activity in Three Strains of Ergot

By E. JOHN STABA, TULLY J. SPEAKER†, and ARTHUR E. SCHWARTING‡

It was observed that the degree of transaminase activity was significantly higher in the alkaloid producing *Pennisetum* strain as compared to the degree of transaminase activity present in the nonalkaloid producing *Claviceps purpurea* (Fries) Tulasne and *Claviceps litoralis* Kawatani strains when they were grown in a defined medium as still cultures.

TRANSAMINASE REACTIONS are known to occur in bacteria, in higher plants, and in animal tissues. The reaction appears to be universal in living organisms. Pyridoxal phosphate is acknowledged to be an essential cofactor in this reaction.



The pseudosclerotia of a strain of ergot grown on a medium containing α-ketoglutaric acid have been reported to contain more than three times as much alkaloid as the accompanying medium. It should be noted, however, that this same strain produces significantly more alkaloids if grown on a medium with succinic acid (1). α-Ketoglutaric acid is known to be an important bridge in the interconversions of carbohydrates and amino acids. With these facts in mind, selected strains of ergot were studied for L-aspartic-glutamic transaminase activity.

EXPERIMENTAL

General Methods.—The following three strains of ergot were studied: (*a*) RCNA-II, a culture of *Claviceps purpurea* (Fries) Tulasne isolated from a high alkaloid-containing sclerotia from rye grown in Connecticut. (*b*) *Litoralis*, a culture of *Claviceps litoralis* Kawatani isolated from a high alkaloid-containing sclerotia from *Elymus mollis* Trin. grown in Japan.[1] (*c*) *Pennisetum*, a culture of ergot

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A portion of this investigation is related to a thesis submitted by E. J. Staba to the School of Pharmacy, University of Connecticut, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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[1] Courtesy of Dr. S. K. Sim, Ontario, Canada, 1958 (Strain K14; 21–23).

from a sclerotia on *Pennisetum typhoideum* Rich. grown in French Equatorial Africa (strain No. 5).

Seed cultures of each of the three strains were grown for 7 days in 500-ml. Erlenmeyer flasks containing 100 ml. of the following medium on a reciprocal shaker (stroke: 1.2 cm.; rate/minute: 100).

Medium	Per Cent
Mannitol	10.0
Methanol	3.0
Succinic acid	1.0
Bacteriological peptone (Nutritional Biochemical Corp.)	0.6
Potassium dihydrogen phosphate	0.2
$MgSO_4 \cdot 7H_2O$	0.1
Distilled water	
Ammonium hydroxide to pH 5.2	

With the exception of the bacteriological peptone all chemicals used in the preparation of the medium were of reagent grade. The medium was sterilized by autoclaving at 15 pounds pressure for 20 minutes.

Approximately 150 mg. of the mycelial seed culture (calculated as dry weight) of each strain was transferred on a standard nichrome wire transfer cylinder into 1-L. Roux bottles containing 100 ml. of the above medium. The Roux bottles were then incubated as still cultures at a temperature of approximately 28°.

Transaminase Determination.—Transaminase was determined manometrically and chromatographically by the amount of glutamic acid formed in an incubation mixture containing pyridoxal phosphate (California Corporation for Biochemical Research), L-aspartic acid, and α-ketoglutaric acid. Unless otherwise indicated, all the solutions in the paragraph below were made in 0.1 *M* potassium phosphate buffer (pH 7.5).

At appropriate time intervals the mycelium contained in one or more Roux bottles was removed from the medium by filtration and thoroughly washed with the buffer. The resulting growth medium, free of mycelium was immediately frozen and subsequently examined for its pH and alkaloid content. The washed mycelium was suspended in 7.5 ml. of pyridoxal phosphate solution (20 mcg./ml.) and incubated in a 50-ml. test tube for 20 minutes at room temperature. At the end of this time, 15 ml. of L-aspartic acid solution (20 μm./ml.) and 7.5 ml. of α-ketoglutaric acid solution (40 μm./ml.) were added to the test tube. The mixture was allowed to react for $1^1/_2$ hours in a constant temperature water bath at 35°. The test tube was then placed in boiling water for 5 to 10 minutes to stop the enzymatic reactions. Four milliliters of 3 *M* sodium acetate buffer (pH 4.8) was added to the mixture and the mixture was filtered. A 1.0-ml. aliquot of the filtrate was immediately used for Warburg analysis, and the remainder of the filtrate was frozen and subsequently examined chromatographically for the presence of glutamic acid and L-aspartic acid. The mycelium resulting from this filtration was transferred quantitatively to a tared disk of aluminum foil and dried to constant weight.

Warburg Procedure.—The procedure used was adapted from the methods of Meister, *et al.* (2), for the manometric determination of glutamic acid. Glutamic acid may be determined in the presence of L-aspartic acid and α-keto acids by inhibiting any possible aspartic decarboxylase enzyme present with trimethylammonium bromide (3).

The following solutions were added to a Warburg flask

Main Compartment	Amount, ml.
Aliquot of incubation mixture	1.0
0.2 *M* Sodium acetate buffer (pH 4.8)	1.0
Trimethylammonium bromide (Eastman Kodak, Technical), 1.31% solution in 0.2 *M* sodium acetate buffer (pH 4.8)	0.5
3 *M* Sodium acetate buffer (pH 4.8)	0.2
Side Arm	
Lyophilized *Clostridium welchii* glutamic acid decarboxylase preparation (NCTC 6785; Worthington Biochemical Corp., N. J.), 15 mg. suspended in 0.2 *M* sodium acetate buffer (pH 4.8)	0.3
Total volume for each Warburg flask	3.0

After equilibrating the Warburg flasks at 38° (Bronwill Warburg UV; stroke: 4 cm.; rate/minute: 110), the 0.3 ml. of enzyme suspension was tipped into the main Warburg compartment and the reaction allowed to proceed to completion as indicated by carbon dioxide evolution. The reaction usually required 40 minutes under the conditions employed. Air was used as the gas phase after it was noted that oxygen uptake was negligible. The corrected value for the μl. of carbon dioxide evolved was determined by the usual procedures (4).

Chromatographic Procedure.—Ten-microliter samples of each of the incubation mixtures was applied to 18 × 24 inch sheets of Whatman No. 1 chromatographic paper together with controls of 0.04 and 0.06-μm. quantities of known L-aspartic acid and glutamic acid. The spotted sheets were allowed to equilibrate for a minimum of 3 hours in an atmosphere produced by phenol-saturated water and were developed in descending technique with water-saturated phenol for periods of 15 hours. After oven drying, the chromatograms were sprayed with 0.1% ninhydrin in acetone and again oven heated to promote color formation. The degree of asymmetry of the resulting chromatographic spots was not sufficiently large to require desalting of the samples.

Using a Photovolt Densitometer (model 52C, Photovolt Corp., New York, N. Y.) absorbances were measured for each mm. along the diameter of the colored spots in the direction of solvent flow and then plotted. The areas under the curves so obtained were measured with a planimeter. For each chromatograph sheet, standard curves were made relating planimeter-measured areas to the known and unknown concentrations of amino acids. These data for the conversion of L-aspartic acid to glutamic acid are presented in Table I.

Alkaloid Analysis.—Two-milliliter portions of each of the filtered medium samples were made alkaline with 0.1 ml. ammonium hydroxide T.S. and extracted three times with 2-ml. portions of ether. The combined ether extracts from each sample were extracted twice with 3-ml. portions of 2.0% tartaric acid solution. To the combined tartaric acid extracts of each culture medium sample was added a 4-ml. portion of *p*-dimethylaminobenz-

TABLE I.—GROWTH DATA, PERCENTAGE TRANSAMINASE ACTIVITY, AND ALKALOID PRODUCTION FROM *Claviceps* CULTURES OF DIFFERENT AGES

Age of Culture, Days	RCNA II[a]			Litoralis[a]			Pennisetum			
	Mycelial Weight, mg.[b]	% T[c] Manometric	% T[c] Chromatographic	Mycelial Weight, mg.[b]	% T[c] Manometric	% T[c] Chromatographic	Mycelial Weight, mg.[b]	% T[c] Manometric	% T[c] Chromatographic	Alkaloid Produced[d]
12	130.2	35.3	38.1	63.2	25.0	20.4	...	...	...	...
14	201.5	42.8	45.6	100.2	28.4	20.4	...	...	...	...
16	110.2	31.5	33.5	137.8	34.1	31.6	13.5	8.8	15.8	slight
19	272.0	30.8	...	93.4	20.8	20.1	12.9	7.0	10.1	12.4
23	292.9	43.3	36.7	303.3	43.4	23.8	24.1	12.0	14.3	8.5
25	229.3	38.6	35.8	187.3	30.7	31.3	14.6	10.2	13.6	27.4
28	268.1	40.9	34.7	421.8	33.8	23.5	12.0	9.3	8.0	20.0
30	364.0	57.2	44.4	271.5	45.5	45.5	17.5	14.8	10.1	49.2
33	336.2	44.9	50.1	700.3	37.8	53.3	8.9	9.4	13.4	54.0
36	510.2	43.0	47.2	439.2	40.0	40.6	16.3	11.7	11.8	8.6
41	332.0	38.4	43.8	562.5	51.5	50.0	23.0	10.8	13.3	76.5
50	397.3	34.5	28.6	533.6	44.0	42.8	53.8	21.4	17.5	26.0

[a] Negative alkaloid production. [b] Dried mycelial weight determined from each incubation mixture. [c] Percentage of L-aspartic acid converted to glutamic acid as determined by manometric and chromatographic procedures for the indicated dry mycelial weight. [d] Mg. % of alkaloids produced in the medium for 10.0 mg. of dried mycelia.

aldehyde reagent (5) and the mixtures were rendered homogeneous by stirring. The volume of each solution was rapidly adjusted to 10 ml., again stirred, and immediately exposed to a mercury vapor lamp for a period of 15 minutes. Immediately after irradiation, the absorbance of the samples was measured in a Bausch and Lomb Spectronic 20 spectrophotometer at 590 mμ. Solutions of U. S. P. Reference Standard Ergonovine Maleate of known appropriate concentrations in 2.0% tartaric acid were similarly treated. No attempts were made to separate or identify the alkaloids from the culture medium.

Table I relates *Pennisetum* alkaloid production to transaminase activity.

TABLE II.—pH OF MEDIUM FROM *Claviceps* CULTURES OF DIFFERENT AGES

Age of Culture, Days	RCNA-II	*Litoralis*	*Pennisetum*
12	4.60	4.45	...
14	4.95	4.60	...
16	4.60	4.66	5.21
19	4.90	4.50	4.60
23	5.10	4.80	4.60
25	4.95	4.62	4.60
28	5.10	4.90	4.60
30	5.25	5.00	4.62
33	5.23	5.18	4.60
36	5.20	5.10	4.62
41	5.31	5.25	4.70
50	5.35	5.30	4.80

RESULTS AND DISCUSSION

In saprophytic cultures, RCNA-II is generally considered to be a nonpigmented, nonalkaloid producing strain; *Litoralis* a dark pigmented, nonalkaloid producing strain; and *Pennisetum* a nonpigmented, alkaloid producing strain of ergot. In all three strains, decrease in pH of the medium was observed which gradually reversed itself with time (Table II).

Table I represents the percentage of L-aspartic acid which is converted to glutamic acid in the incubation mixture for each of these strains as determined by both manometric and chromatographic procedures. In each manometric experiment, known volumes of glutamic acid solution (10 μm./ml.) liberated the expected theoretical amount of carbon dioxide (±5%) with the lyophilized glutamic decarboxylase enzyme.

Examination of Table I will demonstrate that the percentage of L-aspartic acid converted to glutamic acid by *Pennisetum* was less than that converted by the more rapidly growing nonalkaloid producing strains but was markedly greater on a dry mycelial weight comparison.

Alkaloid production of *Pennisetum* still cultures seemed to increase during the time of increased transaminase activity. It was also observed that the nonalkaloid producing strains, at no time during growth, showed evidence of a marked increase in transaminase activity.

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Application of Absorbancy Ratios to the Analysis of Pharmaceuticals I

Theory of the Analysis of Binary Mixtures

By M. PERNAROWSKI†, A. M. KNEVEL, and J. E. CHRISTIAN

The absorbancy ratio technique used in the analysis of binary mixtures has many applications in the pharmaceutical sciences. The theory of this method is discussed, a number of equations are derived, and the advantages and operating characteristics of the technique are presented in this paper.

THERE IS an ever increasing tendency to complexity in pharmaceuticals. Preparations containing two or more ingredients are becoming more common and as a consequence the problems facing the analytical chemist are becoming more complex. Many methods are available for the analysis of such mixtures—some good and others of doubtful value. This paper is concerned with the analysis of binary mixtures by the so-called absorbancy ratio method of analysis. Much neglected, this absorptiometric method has certain qualities that recommend it to the analysts concerned with the determination of complex pharmaceuticals.

This method was first used in Germany at the turn of the century (1). It can be said, in fact, that Hüfner, the originator of this technique, comprehended the fundamental aspects of such analyses at a time when even the validity of Beer's law was in doubt. As with many other types of analyses, the techniques described by Hüfner lay dormant in the literature until well after the second world war. A series of papers appeared at that time dealing with absorbancy ratios themselves and with their application to the analysis of binary mixtures. Schroeder, Wilcox, Trueblood, and Dekker (2) were perhaps the first to tabulate the absorbancy ratio values for a relatively large number of substances of chemical importance. Kuratani (3), Hirt, King, and Schmitt (4), and Bonnier and de'Gauemaris (5) pointed out that such ratios can be used to assess the relative concentrations of the two components in a binary mixture. Moshe Ish-Shalom and his co-workers (6) compared the method as described by Hirt and others (4) with the simultaneous equation method and concluded that the former method gave better results. Pride and Stern (7) derived an equation based on absorbancy ratio values in order to facilitate the colorimetric determination of morphine in the presence of pseudomorphine.

To end the discussion here is to leave much unsaid. The references listed above cover at least the more important aspects of this subject. Many more references are present in current and past journals indicating that this technique and, more important, the ratios themselves, are becoming an important tool in the qualitative and quantitative assessment of a variety of substances of importance to the pharmaceutical sciences.

Many investigators have shown that there are no theoretical limitations inherent in Beer's law but that apparent deviations do occur under experimental conditions. Since this paper concerns itself with the mathematics of the absorbancy ratio technique, it is assumed that this law is obeyed at all wavelengths and at all concentrations. The terminology and symbology used in this paper shall be that suggested by the National Bureau of Standards (8). Hence, absorbancy (A_s) is equal to the product of the absorbancy index (a_s), the cell length (b), and the concentration (c).

CONSTANCY OF THE ABSORBANCY RATIO VALUE

Hüfner (1) was perhaps the first researcher to show that the ratio of two absorbancy values determined on the same solution at two different wavelengths is a constant. From Beer's law and from the spectral characteristics of the hypothetical substance X illustrated in Fig. 1, the constancy of this ratio may be proved mathematically in the following manner. At λ_2, the total absorbancy due to component X (A_{2x}) is given by the following equation

$$A_{2x} = a_2bCx \qquad \text{(Eq. 1)}$$

Similarly, at λ_1, the total absorbancy due to component X (A_{1x}) may be defined as above

$$A_{1x} = a_1bCx \qquad \text{(Eq. 2)}$$

When Eq. 1 is divided by Eq. 2, the following relationship is established

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$$\frac{A_{2x}}{A_{3x}} = \frac{a_2bCx}{a_3bCx} = \frac{a_2}{a_3} = Q{:}2{:}3 \qquad \text{(Eq. 3)}$$

The ratio of two absorbancy values is, therefore, equal to the ratio of two absorbancy index values. Such a ratio is a constant and is designated as the absorbancy ratio. The symbol Q is assigned to this constant along with a specification of the wavelengths at which the measurements were carried out. Hence, $Q{:}2{:}3$ means that the constant has been calculated by dividing the absorbancy determined at λ_2 by that determined at λ_3, the cell and the solution being the same in both cases. There is some rationale for the use of this symbol. Firstly, Hüfner (1) observed the constancy of the ratio calculated from the absorbancies measured at 540 and 560 mμ of a solution of hemoglobin. This ratio was named Hüfner's quotient. Secondly, Zerban and his co-workers (9, 10) used the symbol Q synonymously with absorbancy ratio in their work on coloring matter in sugars. It appears consistent, therefore, to use this symbol and to extend it to include the wavelengths at which the measurements have been carried out.

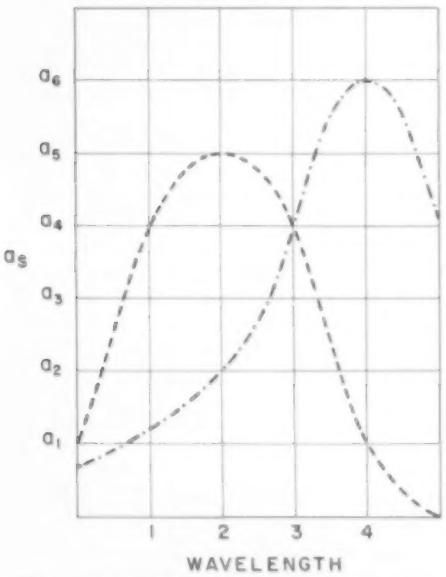

Fig. 1.—Hypothetical spectrophotometric curves for X (- - - - -) and Y (- - - - -) illustrating spectral characteristics necessary for binary Q analysis.

Q ANALYSIS

Q analysis is an abbreviation for absorbancy ratio method of analysis. Such a method of analysis is based on the relationship between the absorbancy ratio value of a binary mixture and the relative concentration of such a mixture. Three equations will be derived below to show the applicability of absorbancy ratios to the analysis of binary mixtures. The spectral characteristics for two hypothetical compounds, X and Y, illustrated in Fig. 1, will be used to clarify the derivation.

At λ_4, the total absorbancy of a mixture containing X and Y (A_4) is equal to the sum of the absorbancies due to X (A_{4x}) and Y (A_{4y}). From Beer's law, however, the total absorbancy due to the components in the mixture may also be defined as follows

$$A_4 = a_6bCx + a_1bCy \qquad \text{(Eq. 4)}$$

Cx and Cy are the concentrations of X and Y, respectively.

In exactly the same way, it may be shown that

$$A_2 = a_2bCx + a_5bCy \qquad \text{(Eq. 5)}$$

Divide Eq. 4 by Eq. 5

$$\frac{A_4}{A_2} = \frac{a_6bCx + a_1bCy}{a_2bCx + a_5bCy} \qquad \text{(Eq. 6)}$$

Assume that b is equal to 1, divide each term by $Cx + Cy$ and substitute Fx for $Cx/(Cx + Cy)$ and Fy for $Cy/(Cx + Cy)$. These latter terms indicate the fraction of the respective components present in the mixture. Therefore

$$\frac{A_4}{A_2} = \frac{a_6Fx + a_1Fy}{a_2Fx + a_5Fy} \qquad \text{(Eq. 7)}$$

The sum of Fx and Fy is equal to one. Hence

$$Fy = 1 - Fx \qquad \text{(Eq. 8)}$$

Substitute $1 - Fx$ for Fy in Eq. 7

$$\frac{A_4}{A_2} = \frac{a_6Fx + a_1(1 - Fx)}{a_2Fx + a_5(1 - Fx)} \qquad \text{(Eq. 9)}$$

Group terms in Eq. 9 and substitute Qo for A_4/A_2, this being the absorbancy ratio for the binary mixture. Care must be exercised in the interpretation of the symbol Qo. Unlike the absorbancy ratio for a pure substance, this symbol implies a variable that depends on the initial concentrations of the two components in the binary mixture. Thus, the initial ratio of X to Y fixes the value of Qo, but because this ratio is fixed for a specific mixture, the degree of dilution of the two substances by some solvent will not alter the value of Qo within the limits of accurate absorptiometric measurements

$$Qo = \frac{Fx(a_6 - a_1) + a_1}{Fx(a_2 - a_5) + a_5} \qquad \text{(Eq. 10)}$$

The equation shown above indicates that a plot of Qo *vs.* Fx is a curve, the extremities of which are given by the expressions a_6/a_2 (the absorbancy ratio for component X) and a_1/a_5 (the absorbancy ratio for component Y). Such a curve may be constructed from a knowledge of the above Q values and also from the Qo values observed for various mixtures of X and Y. This type of analysis must necessarily be graphical. It is possible to use Eq. 10 but a knowledge of four absorbancy index values is required. If such values are determined, the simultaneous equation method may be used and hence destroy any of the usefulness of the absorbancy ratio method of analysis.

Equation 10 may be simplified by taking an isoabsorptive wavelength as the second reference point. Figure 1 indicates that such a point occurs at λ_3. If the derivation is now carried out in a manner similar to the derivation of Eq. 7, the following equation results

$$\frac{A_4}{A_3} = \frac{a_6Fx + a_1Fy}{a_4(Fx + Fy)} \qquad \text{(Eq. 11)}$$

As indicated above, substitute 1 for $Fx + Fy$ and $1 - Fx$ for Fy. Collect terms and rearrange

$$\frac{A_4}{A_3} = \frac{a_6Fx + a_1 - a_1Fx}{a_4} \qquad \text{(Eq. 12)}$$

$$\frac{A_4}{A_3} = \frac{a_6Fx}{a_4} - \frac{a_1Fx}{a_4} + \frac{a_1}{a_4} \qquad \text{(Eq. 13)}$$

$$\frac{A_4}{A_3} = Fx\left[\frac{a_6}{a_4} - \frac{a_1}{a_4}\right] + \frac{a_1}{a_4} \qquad \text{(Eq. 14)}$$

A_4/A_3 is equal to Qo; a_6/a_4 is equal to Qx, the absorbancy ratio for pure X; and a_1/a_4 is equal to Qy, the absorbancy ratio for pure Y. Substitute these symbols into Eq. 14

$$Qo = (Qx - Qy)\,Fx + Qy \qquad \text{(Eq. 15)}$$

Equation 15 is the equation of a straight line having a slope value of $Qx - Qy$ and an intercept value of Qy. The relative analysis of a binary mixture can, therefore, be carried out by determination of the absorbancy ratio values for the pure substances only. More important, Eq. 15 is concentration independent. Analysis of a binary mixture does not, therefore, require careful dilutions or accurate weighings. Similarly, the absorbancy ratios of the pure substances are concentration independent within the limits of accuracy of absorptiometric measurements. Relative analysis of binary mixtures by the absorbancy ratio method is decidedly faster than the simultaneous equation technique but, unlike the latter method, results are in terms of fractions of the total mixture.

If absolute concentrations are desired, the initial weight of the sample must be known, as is true for any analysis. Furthermore, an examination of conditions existing at the isoabsorptive point reveal that Eq. 15 can yield absolute values.

At the isoabsorptive wavelength

$$A_3 = a_4bCx + a_4bCy \qquad \text{(Eq. 16)}$$

Assume that b is equal to 1, then

$$A_3 = a_4(Cx + Cy) \qquad \text{(Eq. 17)}$$

$$Cx + Cy = A_3/a_4 \qquad \text{(Eq. 18)}$$

The total concentration of the two substances can be determined and by combining Eqs. 18 and 15 the absolute concentration of X can be determined. Rearrange Eq. 15

$$Fx = \frac{Qo - Qy}{Qx - Qy} \qquad \text{(Eq. 19)}$$

Since Fx represents the fraction of component X in the binary mixture and A_3/a_4 represents the total weight of X and Y, the concentration of X can be determined from the following equation

$$Cx = \frac{Qo - Qy}{Qx - Qy} \times \frac{A_3}{a_4} \qquad \text{(Eq. 20)}$$

The concentration of Y may be determined in a similar manner or by subtracting the value obtained for Cx from the value obtained for A_3/a_4.

CONCLUSION

Three equations have been derived that may be utilized in the analysis of binary mixtures. The application of these equations to various mixtures will be dealt with in a subsequent paper. For more complete derivations and for a more comprehensive evaluation of the literature, the reader is referred to the original work of the author (11).

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Application of Absorbancy Ratios to the Analysis of Pharmaceuticals II

Practical Aspects of the Analysis of Binary Mixtures

By M. PERNAROWSKI†, A. M. KNEVEL, and J. E. CHRISTIAN

The simultaneous analysis of the components of binary systems may be easily carried out by utilizing the theoretical principles inherent in the absorbancy ratio technique. The limitations of this method of analysis are few and occur because of the failure of Beer's law under extreme experimental conditions or the absence of optimum spectral characteristics for the substances being investigated. The advantages of the method outweigh these limitations and, as a result, the technique may be used in a variety of situations where the control of binary systems is of importance to the analytical chemist.

THE THEORY of the absorbancy ratio method of analysis has been discussed in a previous publication (1). Such theory indicates that a uniform relationship exists between the absorbancy ratio values (calculated from absorbancy readings at two different wavelengths) of binary mixtures containing the same two substances and the relative concentration of such mixtures. Derivations of pertinent equations and a survey of the literature may be found in the publication cited above or in the original work of the authors (2).

The terminology and symbology used in this paper shall be that suggested by the National Bureau of Standards (3). Hence, absorbancy (A_s) is equal to the product of the absorbancy index (a_s), the cell length (b), and the concentration (c) expressed in Gm. per L. The absorbancy ratio value (hereafter referred to as the Q value) is more accurately defined by inclusion of the wavelengths at which absorbancy measurements are carried out. Thus, Q:250:275 refers to the absorbancy ratio value of a solution determined by dividing the absorbancy of this solution at 250 mμ by that at 275 mμ. The term "Q curve" refers to the curve resulting from a plot of Q values *vs.* the relative concentration of one of the two components in a binary mixture.

EXPERIMENTAL

Purity of Solvents and Chemicals.—All chemicals were recrystallized and the observed melting points checked against values reported in the literature. Chloroform, the only organic solvent used in this investigation, was purified by the method described by Riddick and Toops (4).

Apparatus.—Two Beckman model DU spectrophotometers and one Perkin Elmer model 21 infrared spectrophotometer were used in this investigation. These instruments were checked for wavelength accuracy by the methods suggested by the National Bureau of Standards (5) or by the manufacturer (6). The Fisher-Johns melting point apparatus, the balances, and the volumetric glassware necessary to the investigation were calibrated prior to use.

General Procedure for the Analysis of Binary Mixtures.—(*a*) Determine the spectral characteristics of the individual components of the binary mixture. The techniques necessary to obtain these characteristics will vary with the region of the electromagnetic spectrum being utilized in the analysis but, regardless of the nature of the radiant energy, good analytical practice is a necessity in order to produce the best possible spectrum. (*b*) If necessary, locate the isoabsorptive point for the mixture. Methods for locating such a point are given below. (*c*) Choose the two wavelengths to be used in the analysis. These will usually be the wavelength at which one of the two substances exhibits maximum absorption and the isoabsorptive point. (*d*) Calculate the appropriate Q values for the two substances. Absorbancy index values may also be required. (*e*) Determine the Q values of a number of synthetic mixtures. Plot the data (Q *vs.* the per cent of one of the components in the mixture), and if one of the wavelengths used in the analysis is an isoabsorptive point subject the data to the method of least squares (7) to ascertain the equation of the resulting straight line. (*f*) Substitute numerical values into the appropriate equation (1, 2) when applicable.

Location of Isoabsorptive Points.—An isoabsorptive point may be defined as the wavelength at which two dissimilar substances have identical absorbancy index values, the solvent being the same for both substances. The point may be located graphically by superimposing the absorbancy index-wavelength curves of the two substances. Such an approximation is usually inadequate and a point by point determination of the absorbancy index values in the vicinity of the required wavelength is, therefore, necessary. The wavelength at which the two substances have identical absorbancy index values is the isoabsorptive point. The tediousness of the method is at once evident.

The principles inherent in differential spectroscopy provide an alternative method. If the spectrum of a solution of one substance is recorded relative to a solution of another substance (that is, the second solution is the "blank"), the wavelength

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at which an absorbancy reading of zero is observed represents an isoabsorptive point provided that the initial concentrations of the two substances are identical. Both methods were used in this investigation and in only one case did these fail to agree. This is discussed in a proximate portion of this paper.

Analysis in the Presence of an Impurity.—Analytical problems often occur where the concentration of only one of two substances in a mixture is required. The second substance may be an impurity or a spectrally active but physiologically inactive chemical. Such analysis may be carried out conveniently by using absorbancy ratios, provided the spectral characteristics of both substances are known.

The general form of the equation necessary for this type of analysis is given below

$$\frac{A_s}{A_t} = \frac{Qx(1 - Qy/Qo)}{Qx - Qy} \qquad \text{(Eq 1)}$$

A_s, in Eq. 1, is the absorbancy of the pure component being analyzed at the wavelength at which it exhibits maximum absorption, A_t is the absorbancy of the mixture at this same wavelength, Qx is the absorbancy ratio of the substance being analyzed and is determined relative to an isoabsorptive point, Qy is the absorbancy ratio of the impurity, and Qo is the absorbancy ratio of the mixture.

To illustrate the validity of this equation, benzocaine was analyzed in the presence of methylparaben. All absorbancy measurements were carried out on aqeuous solutions of these two substances. The wavelengths chosen for the analysis were 285 mμ (the point at which benzocaine exhibits maximum absorption) and 266.5 mμ (the isoabsorptive point). Q values for benzocaine and methylparaben were determined from absorbancy readings on solutions containing these two substances and substituted into Eq. 1

$$\frac{A_s}{A_t} = 1.157\,(1 - 0.191/Q{:}285{:}266.5)$$

An unknown is analyzed by measuring the absorbancy at 285 mμ (A_t) and at 266.5 mμ and then calculating the Q:285:266.5 value. This equation yields only a corrected absorbancy value for benzocaine at 285 mμ (A_s) and hence a division by 103.3 ± 0.4,[1] the absorbancy index value of benzocaine at this wavelength, is necessary to obtain the concentration of benzocaine in the mixture. The results obtained for mixtures containing these two substances are shown in Table I.

Relative Analysis of Binary Mixtures.—*Graphical or Mathematical Method.*—A plot of Q values *vs.* the per cent of one of the two components in a binary mixture results in a curve. The two wavelengths used in this type of analysis are those that represent the points at which the individual substances exhibit maximum absorption. The equation for this type of curve is given below

$$Qo = \frac{Fx\,(a_{xi} - a_{yi}) + a_{yi}}{Fx\,(a_{xii} - a_{yii}) + a_{yii}} \qquad \text{(Eq. 2)}$$

Qo is the absorbancy ratio of the mixture calculated from absorbancy readings observed at the wavelengths at which the individual components of the binary mixture exhibit maximum absorption. The fraction of the X component in the mixture is represented by the symbol Fx. The absorbancy index values for the X and Y components in the mixture at the first wavelength are symbolized as a_{xi} and a_{yi}. Similarly, the symbols a_{xii} and a_{yii} represent absorbancy index values for the components at the second wavelength.

Where no suitable isoabsorptive point occurs when the spectrum of the individual substances are superimposed, the analysis is best carried out by reference to a previously prepared Q curve. Such a curve is relatively easy to prepare. It is possible to use Eq. 2 in its numerical form but the determination of four absorbancy index values is inherently difficult. Both the mathematical and graphical approaches were used in the analysis of the binary mixture containing benzocaine and tetracaine hydrochloride.

Table I.—Recovery of Benzocaine from Mixtures Containing Benzocaine and Methylparaben

Solution	Methylparaben, % Present	Benzocaine, —Gm./L. × 10^{-4}— Present	Recovered
1	23.1	4.02	4.01
2	20.1	4.02	4.01
3	13.6	3.82	3.87
4	9.5	3.82	3.88
5	18.3	3.62	3.66
Average recovery of benzocaine, %			100.7
Standard deviation, %			±0.9

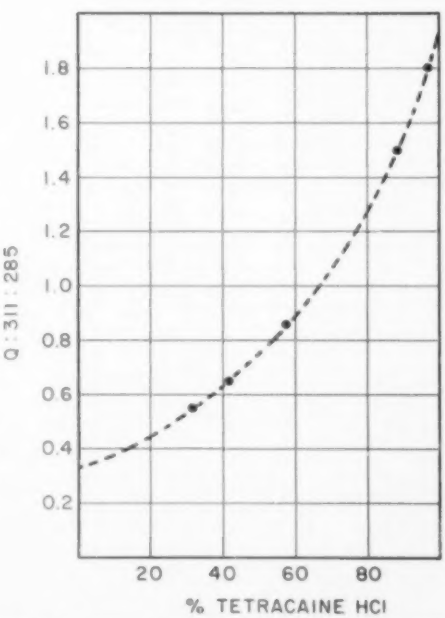

Fig. 1.—Q curve for tetracaine hydrochloride and benzocaine.

[1] Constant values reported in the text of this paper are the average of five separate determinations. The standard deviation is reported after each average value.

In water, benzocaine and tetracaine hydrochloride exhibit maximum absorption at 285 and 311 mμ, respectively. These two wavelengths were chosen for the analysis. The *Q* curve for this binary mixture is shown in Fig. 1. This curve was constructed from data accumulated on binary mixtures containing known amounts of both substances. Solutions of pure benzocaine and pure tetracaine hydrochloride yielded the four absorbancy index values required in the use of Eq. 2 in its numerical form. Values of 103.6 ± 1.1 and 34.7 ± 0.8 were determined for benzocaine at 285 and 311 mμ, respectively. At 311 mμ, the absorbancy index value for tetracaine hydrochloride was found to be 74.7 ± 0.5, while at 285 mμ, the constant was found to be 39.0 ± 0.4. The numerical form of Eq. 2 is given below

$$Q{:}311{:}285 = \frac{40\ Ft + 34.7}{103.6 - 64.6\ Ft}$$

The symbol *Ft* represents the fraction of tetracaine hydrochloride in the binary mixture. The relative quantity of benzocaine in the mixture may be obtained by subtracting the value obtained for tetracaine hydrochloride from one or by using an equation expressed in terms of this component. Five binary mixtures were analyzed by measuring absorbancy values at 285 and 311 mμ, calculating *Q*:311:285, and substituting this value into the above equation. The results of these analyses are shown in Table II. The values reported therein are relative but absolute values can be obtained if the initial step in the analysis represents a weighing.

Table II.—Results of the Analysis of Mixtures Containing Benzocaine and Tetracaine Hydrochloride

Solution	Benzocaine, Present, %	Benzocaine, Found, %	Tetracaine HCl, Present, %	Tetracaine HCl, Found, %
1	3.2	2.9	96.8	97.1
2	58.6	59.2	41.4	40.8
3	10.8	11.5	89.2	88.5
4	42.5	42.8	57.5	57.2
5	68.2	68.8	31.8	31.2

Mathematical Method.—If one of the wavelengths used in the analysis represents an isoabsorptive point, a plot of *Q vs.* the per cent of one of the components in a binary mixture results in a straight line. The equation of such a line is

$$Qo = Fx\,(Qx - Qy) + Qy \qquad \text{(Eq. 3)}$$

The intercept of this straight line is equal to the absorbancy ratio value (*Qy*) of the *Y* component. The slope of this line is equal to the difference between the absorbancy ratio value of component *X* (*Qx*) and that of the *Y* component (*Qy*). The fraction of the *X* component in the mixture is represented by the symbol *Fx* while the absorbancy ratio value of the mixture being analyzed is symbolized as *Qo*. The validity of Eq. 3 was established by analyzing the following binary mixtures:

(*a*) *Acetophenetidin : Caffeine.*—The spectral characteristics of these two substances are shown in Fig. 2. In chloroform, acetophenetidin absorbs ultraviolet radiant energy most strongly at 250 mμ.

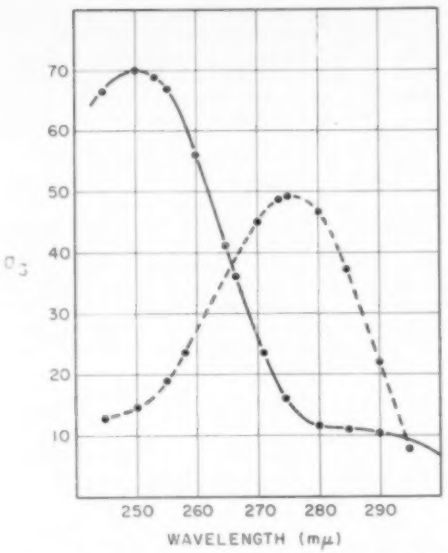

Fig. 2.—Spectrophotometric curves for caffeine (- - - -) and acetophenetidin (———) in chloroform.

Caffeine exhibits maximum absorption at 275 mμ. An isoabsorptive point occurs at 265.5 mμ. The wavelengths chosen for this analysis were 250 and 265.5 mμ. A plot of *Q*:250:265.5 *vs.* the fraction of acetophenetidin in the mixture (*Fa*) results in a straight line. The *Q* curve for this mixture is shown in Fig. 3 and the equation of the line is given below

$$Q{:}250{:}265.5 = 1.532\ Fa + 0.368$$

Such a curve is constructed from data accumulated on mixtures containing known amounts of acetophenetidin and caffeine and the equation of the line is determined by subjecting such data to the method of least squares (7). The amount of caffeine in the mixture may be determined by subtracting the value obtained for acetophenetidin from one or by using an equation similar to that above but expressed in terms of caffeine. The results reported in Table III are relative, but if the initial step in the analysis involved a weighing, absolute values can be calculated.

(*b*) *Phenobarbital:Salicylic Acid.*—Infrared radiant energy was used to analyze this mixture. The instrumental settings on the Perkin Elmer infrared spectrophotometer were those recommended by the manufacturer (6) for quantitative work. Spectra were recorded in the following manner: one 0.5-mm. sodium chloride liquid cell filled with chloroform was inserted in the reference beam and another cell of similar size, filled with the same solvent, was placed in the sample beam. The base line was now recorded over the wavelengths of interest. The sample cell was then removed, cleaned, and filled with the solution to be analyzed. The spectrum for the solution was then recorded over the same wavelength regions in the same direction as the cell filled with solvent only.

The wavelengths chosen for the analysis were

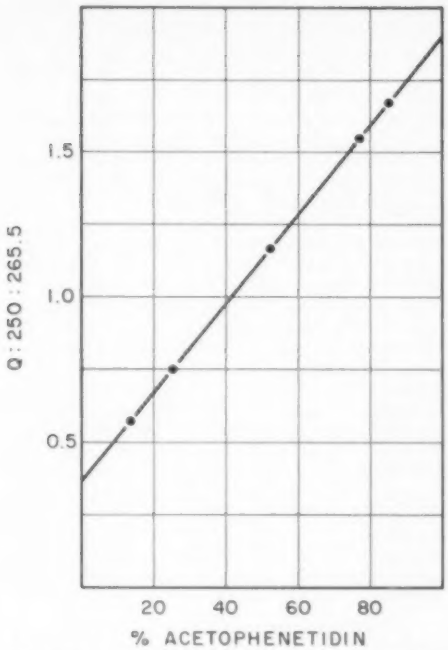

Fig. 3.—Q curve for acetophenetidin and caffeine.

TABLE III.—RESULTS OF THE ANALYSIS OF MIXTURES CONTAINING ACETOPHENETIDIN AND CAFFEINE

Solution	Acetophenetidin		Caffeine	
	Present, %	Found, %	Present, %	Found, %
1	77.0	77.2	23.0	22.8
2	51.9	52.4	48.1	47.6
3	25.4	25.7	74.6	74.3
4	85.5	84.7	14.5	15.3
5	13.6	13.5	86.4	86.5

5.75 μ (the band due to the carbonyl moiety of phenobarbital) and 7.70 μ, the isoabsorptive point (located by superimposing the spectrum of solutions containing identical amounts of solute). A number of solutions containing known amounts of phenobarbital and salicylic acid were prepared and the Q:5.75:7.70 values calculated from absorbancy readings on the infrared spectrum. A plot of Q:5.75:7.70 *vs.* the fraction of phenobarbital in the mixture results in a straight line. The equation of this line is given below

$$Q{:}5.75{:}7.70 = 2.961\ Fp + 0.209$$

Once the fraction of phenobarbital in the mixture (Fp) is known, the amount of salicylic acid may be determined by subtracting this value from one. Alternatively, an equation expressed in terms of salicylic acid may be used to determine the relative concentration of this substance. An analysis is carried out by determining absorbancies at 5.75 and 7.70 μ, making corrections on the basis of background absorption, and using the above equation.

TABLE IV.—RESULTS OF THE ANALYSIS OF MIXTURES CONTAINING PHENOBARBITAL AND SALICYLIC ACID

Solution	Phenobarbital		Salicylic Acid	
	Present, %	Found, %	Present, %	Found, %
1	49.0	50.2	51.0	49.8
2	25.7	25.0	74.3	75.0
3	66.7	67.1	33.3	32.9
4	86.6	86.7	13.4	13.3
5	33.3	33.6	66.7	66.4

The results of the analysis of five mixtures containing phenobarbital and salicylic acid are shown in Table IV.

(*c*) *Sulfathiazole:Sulfapyridine.*—In the last decade, the potassium bromide disk technique has gained favor with many spectroscopists. This technique may be used in conjunction with the absorbancy ratio method of analysis but certain difficulties will be experienced that do not normally occur when analyses are carried out in liquids. It is difficult to prepare two potassium bromide disks of the same thickness and containing identical quantities of active substance. Because of this, it is difficult to locate isoabsorptive points that are necessary for the most favorable use of the absorbancy ratio method.

Two hundred-mesh sulfathiazole was mixed in the proper proportion with 200-mesh potassium bromide. A 250-mg. quantity of the mixture was transferred to a Perkin Elmer potassium bromide pelleting apparatus. The apparatus was subjected to 20,000 pounds per square inch pressure for two minutes using a Carver press. The pellet was removed, placed in the holder provided with the instrument, and the spectrum recorded *vs.* air. Spectra of sulfapyridine and mixtures of sulfathiazole and sulfapyridine were obtained in a manner similar to that above. All pellets used in this investigation weighed between 0.2480 and 0.2520 Gm. and contained between 0.3 and 0.4% active ingredient.

Potassium bromide pellets containing no active ingredient were first studied. The absorption of potassium bromide over the 2 to 15 μ range is essentially linear. The total absorbancy can be expected to vary with the size of the pellet but, for the conditions cited above, the pellets transmitted approximately 75 to 80% of the radiant energy. Six consecutive spectra were recorded for one 0.2480-Gm. pellet. The only operating variable in the above experiment resulted from rotation of the pellet in the holder between each run. The absorbancy values of this pellet at 7.00 μ, one of the wavelengths used in the analysis, varied between 0.108 and 0.113. Since this represents significant absorption, the amount of this absorption must be compensated for in the analysis of binary mixtures or the system treated as a ternary mixture. In this case, the absorbancy due to potassium bromide was subtracted from the total absorbancy observed for all spectrally active substances. In future papers in this series, it will be shown that this system may be treated as a ternary mixture, thus making it unnecessary to carry out the above correction.

The wavelengths chosen for the analysis were 7.00 and 7.55 μ (an approximate isoabsorptive point). The exact nature of the absorption at these wave-

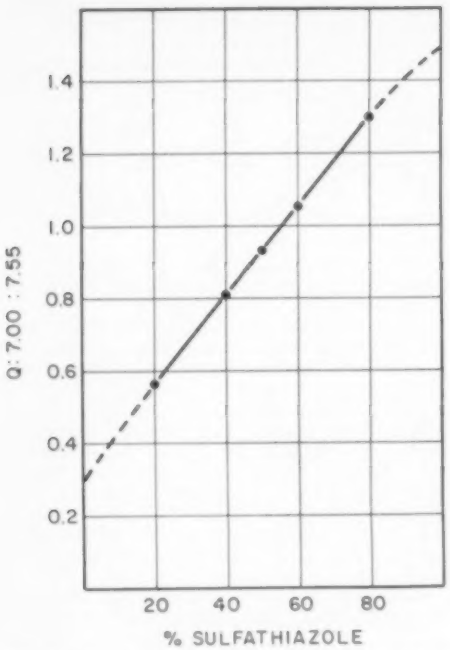

Fig. 4.—*Q* curve for sulfathiazole and sulfapyridine.

lengths is not known but it appears to be associated with sulfonamide-type compounds. A *Q* curve for this mixture was prepared in the usual way and is illustrated in Fig. 4. The curve is not linear, probably indicating that 7.55 μ does not represent a true isoabsorptive point. The curve does have sufficient linearity, however, to allow analysis of mixtures containing from 20 to 80% sulfathiazole. The equation for this portion of the curve is given below

$$Q{:}7.00{:}7.55 = 1.243\ Fs + 0.313$$

Once the fraction of sulfathiazole in the mixture (*Fs*) is determined, the fraction of sulfapyridine may be calculated as indicated in the previous examples. The results of the analysis of five mixtures containing these two substances are shown in Table V.

Table V.—Results of the Analysis of Mixtures Containing Sulfathiazole and Sulfapyridine

Mixture	Sulfathiazole Present, %	Sulfathiazole Found, %	Sulfapyridine Present, %	Sulfapyridine Found, %
1	20.3	19.7	79.7	80.3
2	40.1	41.8	59.9	58.2
3	59.9	61.8	40.1	38.2
4	50.0	50.4	50.0	49.6
5	80.1	79.3	19.9	20.7

Absolute Analysis of Binary Mixtures: Mathematical Method.—If the superimposed spectra of the two substances being analyzed indicate the presence of an isoabsorptive point, an absolute analysis of the mixture is possible provided the absorbancy index value at the isoabsorptive point is known. The general form of the equation necessary for such a determination is given below

$$Cx = \frac{Qo - Qy}{Qx - Qy} \cdot \frac{Ai}{ai} \qquad \text{(Eq. 4)}$$

Cx, in the above equation, refers to the concentration of the *X* component in Gm. per L. of solution, *Qo* is the absorbancy ratio value of the binary mixture, *Qy* is the absorbancy ratio value of the *Y* component, *Qx* is the absorbancy ratio value of the *X* component, *Ai* is the absorbancy of the mixture at the isoabsorptive point, and *ai* is the absorbancy index value at the isoabsorptive point. The wavelength at which one of the substances absorbs radiant energy most strongly and the isoabsorptive point are used in the analysis. The validity of this equation was checked by analyzing three binary mixtures.

(*a*) *Procaine Hydrochloride : Tetracaine Hydrochloride.*—The spectral characteristics of procaine hydrochloride and tetracaine hydrochloride were determined by measuring the absorbancy values of aqueous solutions of these two substances. The wavelengths chosen for the analysis were 311 mμ (the point at which tetracaine hydrochloride absorbs ultraviolet radiant energy most strongly) and 297.5 mμ (the isoabsorptive point). The equation of the straight line resulting from a plot of *Q*:311:297.5 *vs.* the fraction of tetracaine hydrochloride in the mixture (*Ft*) is given below

$$Q{:}311{:}297.5 = 0.682\ Ft + 0.535$$

The absorbancy index value at the isoabsorptive point was found to be 62.0 ± 0.7. Equation 4 may now be written in the numerical form

$$\text{Gm. tetracaine HCl/L.} = \frac{Q{:}311{:}297.5 - 0.537}{0.678} \cdot \frac{A_{297.5}}{62.0}$$

A similar equation may be used to calculate the concentration of procaine hydrochloride in the mixture. Five mixtures were analyzed by measuring absorbancies at 311 and 297.5 mμ. The results of such analyses are shown in Table VI.

Table VI.—Results of the Analysis of Mixtures Containing Tetracaine and Procaine Hydrochlorides

Solution	Tetracaine HCl, Gm./L. × 10^{-4} Taken	Tetracaine HCl, Gm./L. × 10^{-4} Recovered	Procaine HCl, Gm./L. × 10^{-4} Taken	Procaine HCl, Gm./L. × 10^{-4} Recovered
1	2.25	2.26	2.72	2.71
2	1.50	1.50	3.48	3.47
3	3.00	3.04	1.99	1.93
4	2.52	2.54	2.52	2.47
5	0.75	0.74	4.23	4.28
Average recovery, %		100.3		99.0
Standard deviation, %		±1.0		±1.6

(*b*) *Benzocaine : Procaine Hydrochloride.*—The spectral characteristics of these two substances are shown in Fig. 5. The wavelengths chosen for the analysis were 285 mμ (the point at which benzocaine exhibits maximum absorption) and 311 mμ (the isoabsorptive point). The isoabsorptive point was located by determining the absorbancy index values

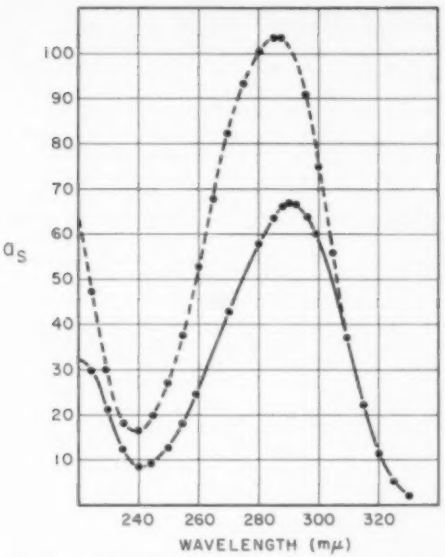

Fig. 5.—Spectrophotometric curves for benzocaine (- - - -) and procaine hydrochloride (———) in water.

of the two substances at several wavelengths on either side of 311 mμ. The differential method for locating this point failed in this case because the slopes of the spectra of benzocaine and procaine hydrochloride are essentially the same in the vicinity of 311 mμ. Absorbancy values will be essentially zero at several wavelengths when the differential technique is used. The absorbancy index value at the isoabsorptive point was found to be 31.9 ± 0.3. The equation of the straight line resulting from a plot of Q:285:311 *vs.* the fraction of benzocaine in the mixture (Fb) is given below

$$Q:285:311 = 1.16\ Fb + 2.03$$

Numerical values may now be substituted into Eq. 4

$$\text{Gm. benzocaine/L.} = \frac{Q{:}285{:}311 - 2.03}{1.16} \cdot \frac{A_{311}}{31.9}$$

Five mixtures were analyzed by measuring absorbancies at 285 and 311 mμ. The results of such analyses are shown in Table VII. The absolute concentration of procaine hydrochloride may be determined by using an equation similar to that shown above.

TABLE VII.—RESULTS OF THE ANALYSIS OF MIXTURES CONTAINING BENZOCAINE AND PROCAINE HYDROCHLORIDE

Solution	Benzocaine, Gm./L. × 10^{-3} Taken	Benzocaine, Gm./L. × 10^{-3} Recovered	Procaine HCl, Gm./L. × 10^{-3} Taken	Procaine HCl, Gm./L. × 10^{-3} Recovered
1	3.01	3.03	2.45	2.46
2	2.01	1.99	3.75	3.78
3	2.41	2.45	3.25	3.23
4	1.60	1.63	4.24	4.27
5	3.41	3.46	2.00	1.96
Average recovery, %		101.0		99.9
Standard deviation, %		±1.2		±1.2

(*c*) *Brucine : Strychnine.*—Solutions of the pure substances in chloroform were prepared and the infrared spectrum recorded over the 2 to the 16 μ wavelength region. The general techniques used were the same as those described for the phenobarbital-salicylic acid mixture. The two wavelengths chosen for the analysis were 7.78 μ (due to a C—O—C asymmetrical stretch) and 6.83 μ, the isoabsorptive point (due to a methyl asymmetrical bend). The isoabsorptive point was located by superimposing spectra in a manner similar to that discussed above.

To check the location of the isoabsorptive point and to determine an apparent absorbancy index value at this wavelength, solutions of both brucine or strychnine were prepared and absorbancy values at 6.83 μ determined graphically. Such values were plotted *vs.* the concentration of the substance in Gm. per 100 ml. of chloroform. The equation of the resulting straight line, determined by the method of least squares, is given below

$$A_{6.83} = 0.371\ Cs + 0.010$$

Cs, in the above equation, is equal to the concentration of strychnine expressed in Gm. per 100 ml. of chloroform. The equation for brucine is given below

$$A_{6.83} = 0.370\ Cb + 0.003$$

Cb, in this case, is equal to the concentration of brucine expressed in Gm. per 100 ml. of chloroform. Because the slopes of the two lines are essentially equal, 6.83 μ must represent an isoabsorptive point. The intercept values indicate that Beer's law is obeyed for the concentration range studied (300–600 mg. solute per 100 ml. of chloroform). The apparent absorbancy index value for these two substances at the isoabsorptive point was taken as 0.371.

The equation of the straight line resulting from a plot of Q:7.78:6.83 *vs.* the fraction of brucine (Fb) in the mixture is given below

$$Q{:}7.78{:}6.83 = 1.096\ Fb + 0.612$$

Equation 4 may now be written in the numerical form

$$\text{Gm. brucine/100 ml. chloroform} = \frac{Q{:}7.78{:}6.83 - 0.612}{1.096} \cdot \frac{A_{6.83}}{0.371}$$

A similar equation may be used to calculate the concentration of strychnine in the mixture. Five mixtures were analyzed by graphically determining absorbancy values at 7.78 and 6.83 μ. The results of such analyses are given in Table VIII.

DISCUSSION

An examination of the results in Tables I to VIII indicates that this technique is capable of yielding very satisfactory results. The standard deviation for the average recovery of the individual components in a binary mixture approximates 1% when analyses are carried out in the ultraviolet region of the electromagnetic spectrum. This value in-

Table VIII.—Results of the Analysis of Mixtures Containing Brucine and Strychnine

Solution	Brucine, Gm./100 ml. Taken	Brucine, Gm./100 ml. Recovered	Strychnine, Gm./100 ml. Taken	Strychnine, Gm./100 ml. Recovered
1	0.4810	0.4942	0.1196	0.1171
2	0.2000	0.2000	0.4014	0.4140
3	0.3009	0.3129	0.3024	0.2930
4	0.4016	0.3986	0.2007	0.2073
5	0.1212	0.1233	0.4812	0.4880
Average recovery, %		101.5		100.5
Standard deviation, %		±1.9		±3.0

creases to 2 to 3% when analyses are carried out in the infrared region. This, however, does not constitute unsatisfactory precision particularly if the precision of conventional infrared techniques are taken into consideration. The errors encountered herein, however, do not truly reflect the accuracy of the absorbancy ratio method. The final accuracy will depend on a number of factors that might be enumerated briefly as accuracy of the absorbancy measurement, relative concentrations of the active ingredients in and nature of the pharmaceutical being analyzed, and spectral characteristics of the components in the binary mixture.

Accurate absorbancy measurements are essential to all types of analyses requiring the use of radiant energy. A maximum of accuracy is achieved by making these measurements at a wavelength at which maximum absorption occurs. A complication occurs, however, when the absorbancy ratio method is used. Theoretically, the slope of a Q curve is equal to the difference of the Q values of the pure substances present in the binary mixture provided one of the wavelengths used in the analysis represents an isoabsorptive point. In actual practice, this is not always true. Thus, the Q:285:266.5 values for benzocaine and methylparaben were found to be 1.405 ± 0.004 and 0.139 ± 0.007, respectively. The slope of the Q curve should be equal to the difference of the above values while the intercept should be equal to 0.139. When a number of mixtures were prepared and the Q:285:266.5 values determined, the equation of the resulting straight line as determined by the method of least squares (7) indicated that the intercept value was 0.191. This implies that deviations occur near the lower end of the Q curve. This, however, is not surprising. Absorbancy readings for methylparaben at 285 mμ are very low irrespective of the concentration of the solution being studied (approximately 0.04). Such values can scarcely be considered accurate and hence the accuracy of the Q:285:266.5 value is poor. This example represents an extreme case. Most other mixtures analyzed tended to approximate more closely theoretical conditions. On the basis of the work reported herein, accuracy and precision can be obtained only by determining the Q values of mixtures containing known amounts of the substances being analyzed and subsequently subjecting the results to the method of least squares.

The pharmaceutical form of the binary mixture is another factor that will influence the accuracy of the analysis. Preliminary operations are an inherent part of any analysis and, therefore, are not basic to this method. More important, however, is the proportion of the active ingredients in the pharmaceutical. Thus, a 50–50 mixture would yield far more accurate results than, for example, a 94–6 mixture. In the latter case, the precision with which the minor ingredient could be determined would be very low. The limiting factor here is, of course, the spectral characteristics of the two components. Cases can probably be found where the absorption intensity of the six parts of the minor component are much greater than those of the major component. There is no reason to suppose, therefore, that the accuracy and precision in this case would be less than that reported in this paper.

The spectral characteristics of the two substances being analyzed play a part in this technique only insofar as they influence the slope of the Q curve. This is evident from an examination of Figs. 2 and 5. In the former figure, the two substances (acetophenetidin and caffeine) exhibit maximum absorption at wavelengths 25 mμ apart. In the later case, the two substances (benzocaine and procaine hydrochloride) exhibit absorption maxima at almost the same wavelength. The position of the peaks is, therefore, of no consequence but the absorption intensity at the two wavelengths used in the analysis is of importance because it affects the slope of the Q curve. The closer the slope value is to zero, the less accurate the analysis. The steeper the slope, the better the accuracy of the analysis, but even here a limit is reached by virtue of low absorbancy readings. The lowest slope value encountered in this investigation was 0.682 (for procaine and tetracaine hydrochlorides). The accuracy and precision encountered with this mixture was quite good but it is doubtful if the slope value could be decreased much below this figure without sacrificing precision and accuracy. As a generalization, therefore, the superimposed spectra of the two substances should show an isoabsorptive point and the absorption intensities of the substances should be such that a maximum slope value is obtained.

As a final consideration, the composite curve of the two substances has an influence on the accuracy of the analysis. Measurements appear to be made on slopes of spectral curves. This is not always the case since composite curves have little relationship to the characteristics of the individual substances. A moments reflection will indicate that an optimum condition will exist where absorbancy readings on either side of a particular wavelength differ but slightly. When this occurs, the mixture being analyzed represents an ideal binary system. Except on theoretical grounds, it is impossible to specify optimum conditions primarily because the spectral characteristics of the substances encountered by the analyst are extremely variable. The easiest way to determine if a particular analysis is feasible is to obtain the spectrum of the mixture being analyzed. For example, if a 50–50 mixture of A and B is to be analyzed and the spectral characteristics of the mixture indicate that the absorbancy readings at the two wavelengths to be used in the analysis can be determined accurately, then there is no reason for the absorbancy ratio technique to yield results less accurate and precise than those reported in this paper.

The experimental portion of this paper described

the analysis of eight binary mixtures by applying four basic equations in the final step of the procedure. All are based on the relationship between the absorbancy ratio value and the relative concentration of a binary mixture. The first of these, Eq. 1, may be discarded since Eqs. 3 and 4 yield the same type of results. Equation 2 may be used but the analysis is limited by the need of previously prepared Q curves. Although this may constitute good analytical practice, the use of graphs is often inconvenient. It is well to remember that portions of this type of curve may be used under certain circumstances. Assume that a 50–50 mixture is required in a particular granulation. The absorbancy ratio for such a mixture might be, for example, 2.00. Even though a plot of Q values *vs.* the relative concentration of the mixture does not constitute a straight line, limits of, for example, 1.98 to 2.02 can be established and, consequently, the mixture can be controlled. This was illustrated in the case of the analysis of sulfathiazole-sulfapyridine mixtures. Since the absorbancy ratio method requires no accurate weighings or dilutions, the uniformity of the granulation can be established rapidly.

If the initial weight of the mixture is known and an isoabsorptive point is used in the final measurement, then Eq. 3 can be used to provide the analyst with the absolute concentration of the individual components in the binary mixture. Once the weight of the mixture is established, however, no absolute dilutions are required, that is, graduated cylinders provide the necessary dilution accuracy. If, however, the weight of the initial mixture is not known or difficult to obtain (for example, the quantities obtained are small or the sample is processed in such a way that the mixture is never recovered in the crystalline form), then Eq. 4 must be used. For this type of analysis, accurate dilutions are required.

The absorbancy ratio method of analysis places no restrictions on the nature of the radiant energy used in the final measurements. In fact, the technique should be applicable to any case where a relationship similar to that existing between absorbancy and wavelength exists. The accuracy of the analysis will vary, of course, from region to region but this is due primarily to experimental and instrumental rather than to theoretical limitations.

CONCLUSIONS

The absorbancy ratio method of analysis is capable of yielding good results if used with caution and common sense. It should be of great importance to the analyst interested in the analysis of various products containing two active ingredients. This method is not a panacea but it does simplify and increase the speed of analysis of various mixtures of importance to the pharmaceutical industry. Part IV in this series of papers will show that absorbancy ratios can be used in the analysis of mixtures containing three active ingredients.

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Application of Absorbancy Ratios to the Analysis of Pharmaceuticals III

Simultaneous Analysis of Aminophylline and Phenobarbital

By F. YOKOYAMA and M. PERNAROWSKI

The principles inherent in the absorbancy ratio technique are applied to the analysis of binary mixtures containing aminophylline or theophylline and phenobarbital. The analysis is carried out without prior separation of the components of the mixture and is applicable to commercial preparations containing these substances.

THE DIURETIC PROPERTIES of theophylline were first observed over fifty years ago. Since that time, this drug and aminophylline have been used either alone or in combination with phenobarbital for the treatment of conditions such as hypertension, bronchial asthma, and cardiac and nephrotic edema. It is these combinations of theophylline or aminophylline and phenobarbital that must be subjected to anal-

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ysis by the control chemist. Even though several procedures have been published for such mixtures, the problems associated with such analyses have not been completely resolved. Hyatt (1) described a method that was later subjected to collaborative study (2). His method, which is based on spectrophotometric measurements and involves separation of the active ingredients by extraction, is the prescribed method of the A.O.A.C. (3). Griffenhagen and Brady (4), Bhattacharya and Banerjee (5), and David (6) proposed argentiometric and gravimetric procedures for aminophylline and phenobarbital combinations. Bartilucci and Discher (7) determined the active ingredients by differential titrimetry in either water or alcohol. Several of these methods, as well as a differential nonaqueous titrimetric procedure, were investigated in this laboratory. The spectrophotometric method described by Hyatt appeared to be the best but involved tedious extractions. This work indicated, however, that a simultaneous analysis of aminophylline and phenobarbital was possible if the techniques described by Pernarowski, Knevel, and Christian (8) were utilized.

The theory of this absorbancy ratio method of analysis is discussed in detail in the above paper. Briefly, a plot of absorbancy ratio values *versus* the fraction of one of the two components in a binary mixture is a straight line if one of the wavelengths used in the analysis represents an isoabsorptive point. The equation of this straight line is easily determined and provides the basis for the subsequent determination of the individual components in the binary mixture.

The terminology and symbology used in this paper shall be that suggested by Pernarowski, Knevel, and Christian (8). Thus, the term "Q value" is an abbreviation for "absorbancy ratio value." The ratio itself is determined by dividing the value of the measured absorbancy at one wavelength by that measured at another wavelength, the cell and the solution being the same for both measurements. A "Q curve" illustrates the relationship between the Q values of particular binary mixtures and the fraction of one of the two components in such mixtures. Other spectrophotometric terms and symbols used in this paper are those described in a letter circular published by the National Bureau of Standards (9).

EXPERIMENTAL

Apparatus.—Beckman model DU spectrophotometer; Shell precision dual titrometer; shielded glass electrode, Beckman No. 41262; calomel electrode, sleeve type, Beckman No. 43-908; Fisher-Johns melting point apparatus, calibrated against U. S. P. melting point standards.

Reagents.—Chloroform, reagent grade. Methanol, Analar grade. Nitric acid C.P. Aminophylline U. S. P.; the sample was assayed by the U. S. P. XVI method (10) and was found to contain, on the basis of five determinations, 82.3 ± 0.4% theophylline. Phenobarbital, recrystallized from aqueous ethanol; the sample melted between 174.5 and 175.5° (corrected) and assayed 99.7% by the method developed in this laboratory (11). Theophylline, recrystallized from ethanol; the sample melted between 270 and 271° (corrected) and assayed 101.1% by the method described in the U. S. P. XVI for aminophylline (10). Ferric ammonium sulfate T. S. Ammonia T. S. Hydrochloric acid, diluted, U. S. P. Silver nitrate, 0.1 *N*. Ammonium thiocyanate, 0.1 *N*. Potassium hydroxide, 0.1 *N* in anhydrous methanol. Thymol blue indicator, 0.5% in anhydrous methanol. Borate buffer, pH 9.5 (12); this solution was prepared by dissolving 6.185 Gm. of reagent grade boric acid and 7.455 Gm. of reagent grade potassium hydroxide in sufficient distilled water to make 500 ml., adding 350 ml. of carbonate-free 0.1 *N* sodium hydroxide, and then sufficient distilled water to make 1 L. of solution.

Spectral Characteristics of Phenobarbital.—Solutions of phenobarbital in borate buffer of pH 9.5 showed absorption maxima at 240 mμ and were stable for twenty-four hours as previously reported (12).

Spectral Characteristics of Aminophylline.—Solutions of aminophylline in borate buffer of pH 9.5 exhibited maxima at 274.5 mμ and were stable over a twenty-four-hour period. When the spectrum of aminophylline was compared with that of theophylline, it was evident that the ultraviolet absorption was due solely to the theophylline portion of the molecule (see Fig. 1). This fact was further substantiated when ethylenediamine itself failed to exhibit any absorption between 235 and 300 mμ as well as causing no alteration in the spectral characteristic of theophylline. The two curves, as shown in Fig. 1, were identical with respect to shape but failed to coincide completely because of the differences in the concentration of the ultraviolet absorbing component theophylline. However, when the weight of aminophylline was altered so as to contain an equivalent amount of theophylline, the two were then found to be completely superimposable.

Location of the Isoabsorptive Point.—The isoabsorptive point for phenobarbital and theophylline was located by comparing spectrophotometrically a 0.001% solution of phenobarbital (sample solution) with a 0.001% solution of theophylline (blank solution). On the basis of accumulated data, the isoabsorptive point for these compounds occurred at 252.5 mμ. This was further substantiated by determining the absorbancy index values of phenobarbital and theophylline at this wavelength. The former value, on the basis of 19 determinations, was equal to 23.7 ± 1.0 while the latter value, on the basis of 27 determinations, was found to be 23.6 ± 0.1. The details associated with the location of isoabsorptive points are given elsewhere (8).

Procedure.—Weigh and powder twenty tablets. Accurately weigh an aliquot representing approximately 100 mg. of aminophylline, add 100 ml. of

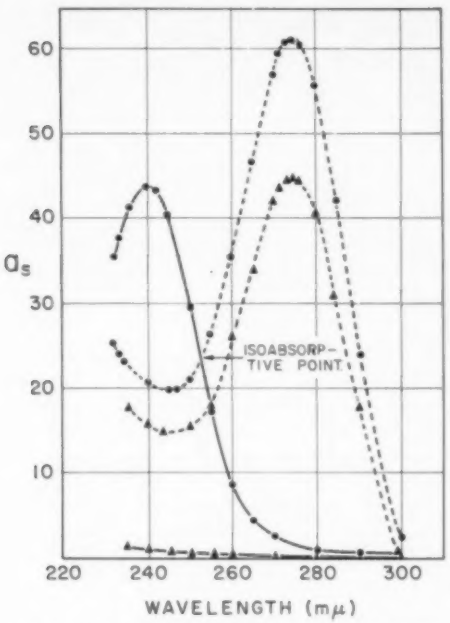

Fig. 1.—Spectrophotometric curves for phenobarbital (—●——●—), theophylline (- -●- -●- -), aminophylline (- ▲ - - ▲ - -), and ethylenediamine (—▲——▲—).

borate buffer solution, and stir electromagnetically for thirty minutes. Filter through Whatman No. 1 filter paper into a 1,000-ml. volumetric flask, wash the residue with four additional 25-ml. portions of the buffer solution, and make up to volume with the same solvent. Take a 10-ml. aliquot of the solution and dilute to 100 ml. with the buffer. Measure the absorbancy of the solution at 252.5 and 274.5 mμ using borate buffer as the blank. Calculate the concentration of phenobarbital and theophylline by substituting the observed values into the following equation

$$\text{Gm. theophylline/L.} = \frac{Q{:}274.5{:}252.5 - 0.0451}{2.60} \times \frac{A_{252.5}}{a_{252.5}} \quad \text{(Eq. 1)}$$

Q:274.5:252.5 in the above equation is the absorbancy ratio value of the solution calculated by dividing the observed absorbancy at 274.5 mμ by that observed at 252.5 mμ. The absorbancy index value of both phenobarbital and theophylline at 252.5 mμ ($a_{252.5}$) is equal to 23.7 ± 0.7 on the basis of 46 determinations. The numerical values of the intercept and the slope of the Q curve are 0.0451 and 2.60, respectively. The concentration of phenobarbital is obtained by subtracting the weight of theophylline from the total weight of active components ($A_{252.5}/a_{252.5}$).

DISCUSSION

The spectral characteristics of phenobarbital, theophylline, ethylenediamine, and aminophylline are illustrated in Fig. 1. These characteristics indicated that binary mixtures containing phenobarbital and either aminophylline or theophylline could be analyzed by applying the principles described by Pernarowski, Knevel, and Christian (8). The first criterion cited by these authors for the use of the absorbancy ratio technique was the presence of an isoabsorptive point when the spectra of the substances being analyzed are superimposed. Such a point does in fact occur when the spectra of phenobarbital and theophylline are superimposed. This wavelength, 252.5 mμ, and the wavelength at which theophylline exhibits maximum absorption, 274.5 mμ, were chosen for the analysis. This choice of wavelengths results in optimum conditions, the slope value of the Q curve in this case being somewhat greater than if 240 mμ, the point at which phenobarbital exhibits maximum absorption, was chosen as the second wavelength. In addition to the above, ethylenediamine exhibits little absorption at 274.5 mμ. This implies that only the theophylline portion of aminophylline absorbs ultraviolet radiant energy. The proposed method of analysis results, therefore, in a direct measure of the therapeutically active moiety "theophylline" actually present in the labeled amount of aminophylline. The determined quantity of anhydrous theophylline can then be converted to an equivalent weight of aminophylline on the basis of established molecular relationships.

A plot of Q:274.5:252.5 values *vs.* the fraction of theophylline in various synthetic mixtures of phenobarbital and theophylline indicated that a linear relationship existed between the two variables over the entire concentration range of 0 to 100% theophylline (see Fig. 2). Although this Q curve is of theoretical interest, commercial preparations rarely contain less than 75% or more than 95% aminophylline, and consequently only that portion of the Q curve marked off as the area of significance and

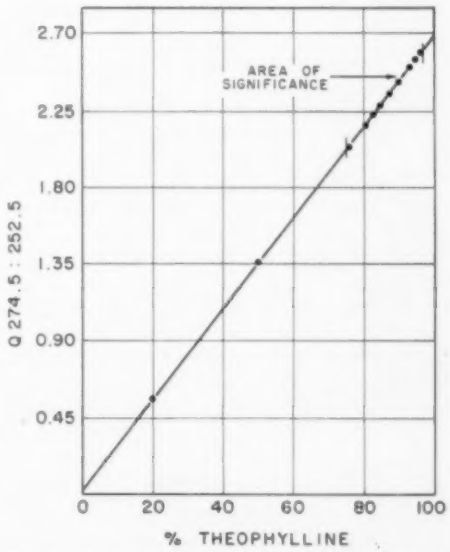

Fig. 2.—Q curve for theophylline and phenobarbital.

representing 74 to 97% theophylline was studied in detail. On the basis of 27 solutions containing both phenobarbital and theophylline, the equation of this portion of the Q curve was found to be

$$Q{:}274.5{:}252.5 = 2.60\ Ft + 0.0451 \quad \text{(Eq. 2)}$$

Ft, in the above equation is equal to the fraction of theophylline present in the binary mixture. This equation yields only relative values. If absolute values are required, Eq. 1 must be used.

To test the overall accuracy and precision of the proposed method of analysis, several synthetic mixtures of known composition were prepared and analyzed. The results of these analyses are shown in Tables I and II and indicate that the major component can be determined with a high degree of accuracy and precision. Phenobarbital, on the other hand, cannot be evaluated with the same degree of precision, the standard deviation being as high as 3% in certain instances. This, however, is a percentage figure and must be judged in relation to the speed and ease of anaylsis and to the quantity of substance being analyzed. The relative proportion of phenobarbital to major component is small and slight discrepancies in absolute recoveries will result in relatively large percentage differences. As an example, a variation of 0.05 mg. of phenobarbital from a mean value of 1.50 mg. will result in a 3.3% deviation from the mean. Absolute differences are, therefore, quite small and not particularly alarming.

Table I.—Results of the Analysis of Synthetic Mixtures Containing Aminophylline and Phenobarbital

Mixture[a]	Aminophylline,[b] mg. Taken	Theophylline, mg. Found	Phenobarbital, mg. Taken	Phenobarbital, mg. Found
1	10.7	8.88	1.57	1.62
	10.7	8.88	1.57	1.62
2	10.2	8.41	1.51	1.55
	10.2	8.41	1.51	1.51
3	10.1	8.35	1.52	1.57
	10.1	8.36	1.52	1.56
4	10.1	8.39	1.53	1.61
	10.1	8.38	1.53	1.54
	Average % theophylline in aminophylline, 82.8 ± 0.2		Average % recovery, 102.6 ± 1.6	
5	10.7	8.86	3.14	3.24
	10.7	8.87	3.14	3.27
6	10.2	8.38	3.03	3.13
	10.2	8.38	3.03	3.17
7	10.1	8.41	3.03	3.02
	10.1	8.41	3.03	3.02
8	10.1	8.36	3.07	3.07
	10.1	8.36	3.07	3.07
	Average % theophylline in aminophylline, 82.8 ± 0.4		Average % recovery, 101.8 ± 2.2	

[a] Mixtures 1–4 simulate commercial preparations containing 1½ gr. aminophylline and ¼ gr. phenobarbital. Mixtures 5–8 are equivalent to commercial preparations containing 1½ gr. aminophylline and ½ gr. phenobarbital. [b] The aminophylline used in the preparation of these mixtures analyzed 82.3 ± 0.4% by the U. S. P. XVI method.

Six samples of commercial tablets were analyzed by the proposed method and, where possible, by the procedure reported by David (6). The latter method involves a gravimetric determination of the phenobarbital in the mixture. Rather than resorting to such a technique, the method was modified so as to permit a titrimetric determination of the phenobarbital by the procedure described by Chatten (11). David's method tended to give slightly lower recoveries for the two components of the mixture, was operationally more difficult, and was not applicable to several of the commercial preparations analyzed. The slight discrepancies in recoveries between the two techniques may be due to a combination of several phenomena observed during various stages of the control method. It is inherently difficult to filter the ammoniacal solution of the tablet mass and this, in itself, may result in lower recoveries of both constituents. In certain cases, emulsions formed at the chloroform-water interface during the phenobarbital extraction step and may partly explain the lower recoveries of this substance. Silver theophyllinate itself is slightly soluble in ammoniacal solution and this factor may account for lower theophylline recoveries from mixtures (13).

Table II.—Results of the Analysis of Synthetic Mixtures Containing Theophylline and Phenobarbital

Mixture[a]	Theophylline, mg. Taken	Theophylline, mg. Found	Phenobarbital, mg. Taken	Phenobarbital, mg. Found
1	8.01	7.87	1.50	1.46
	8.01	7.89	1.50	1.40
2	8.18	8.13	1.51	1.53
	8.18	8.16	1.51	1.55
3	8.01	7.99	1.55	1.55
	8.01	7.97	1.55	1.53
4	8.06	8.06	1.53	1.48
	8.06	8.06	1.53	1.48
	Average % recovery, 99.4 ± 0.7		Average % recovery, 98.3 ± 3.0	
5	8.01	7.89	3.00	2.82
	8.01	7.89	3.00	2.91
6	8.18	8.15	3.02	3.03
	8.18	8.16	3.02	3.02
7	8.01	8.02	3.09	3.07
	8.01	8.02	3.09	3.07
8	8.06	8.09	3.07	3.09
	8.06	8.09	3.07	3.09
	Average % recovery, 99.7 ± 0.8		Average % recovery, 98.9 ± 2.3	

[a] Mixtures 1–4 simulate commercial preparations containing 1½ gr. aminophylline and ¼ gr. phenobarbital. Mixtures 5–8 are equivalent to commercial preparations containing 1½ gr. aminophylline and ½ gr. phenobarbital.

The results in Table III indicate that the proposed method is capable of yielding the same level of precision as that observed for synthetic mixtures. Tablet excipients such as talc, starch, lactose, and magnesium stearate do not interfere with the determination of the active ingredients. Compressed tablets (samples A, B, and C) were easily and quickly analyzed. Coated tablets, on the other hand, caused some difficulties because these contained water-soluble dyes in the outer coat (samples E and F). In these two cases, the method was modified slightly so as to allow for the removal of this outer coat by washing the tablets with distilled water.

The proposed method of analysis appears to be preferable to the methods described in the literature. It involves no solvent-solvent extractions, the manipulative techniques are easily mastered, and the accuracy and precision is perhaps greater than or at least equal to that obtainable by methods in current use.

TABLE III.—RESULTS OF THE ANALYSIS OF COMMERCIAL PREPARATIONS CONTAINING AMINOPHYLLINE AND PHENOBARBITAL

Sample	Aminophylline mg./Tablet Labeled	Theophylline mg./Tablet Found[a]	Theophylline mg./Tablet Found[b]	Phenobarbital mg./Tablet Labeled	Phenobarbital mg./Tablet Found[a]	Phenobarbital mg./Tablet Found[b]
A	97.2	77.6 ± 0.7	77.5	16.2	15.7 ± 0.3	15.6
B	97.2	71.2 ± 0.3	69.6	32.4	28.4 ± 0.4	28.1
C	97.2	77.3 ± 0.6	...	16.2	16.4 ± 0.7	...
D	97.2	73.1 ± 0.6	75.8	16.2	16.6 ± 0.2	...
E	97.2	80.0 ± 0.2	...	16.2	16.5 ± 0.4	...
F	194.4	160.8 ± 0.9	163.2	32.4	34.4 ± 0.9	34.0

[a] Each product was analyzed five times by the method described in the text. The averages and standard deviations of such analyses are reported in this column. [b] Where possible, comparative determinations were carried out using a modification of the method described by David (3). The averages of duplicate determinations are reported in this column.

SUMMARY

A spectrophotometric technique has been developed for the simultaneous determination of mixtures containing aminophylline and phenobarbital. This method is rapid, the manipulative techniques are simple, and the accuracy is equal to that inherent in published procedures.

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Pharmacology of Bunamiodyl I

Toxicity Studies

By IRVING LEVENSTEIN, ANNE WOLVEN, and ARNOLD URDANG†

The acute and chronic toxicity of a synthetic cholecystographic agent, bunamiodyl, 3-(3-butyrylamino-2,4,6-triiodophenyl)-2-ethyl sodium acrylate, was determined in rats and dogs. The LD_{50} was found to be 2.75 Gm. per Kg. of rat, and daily feedings of the material on the basis of 750 mg. per Kg. of rat caused no toxicity. Dogs receiving 1 Gm. of the test material per day for a two-week period showed no toxic symptoms and no damage to their kidneys.

IN THE SEARCH for synthetic cholecystographic agents, bunamiodyl,[1] 3-(3-butyrylamino-2,4,6-triiodophenyl)-2-ethyl sodium acrylate, was selected since it exhibited excellent opacity and a low incidence of side effects associated with these agents. A dose of 4.5 Gm. is necessary to obtain good gallbladder opacity in the human. It was necessary to carry out studies to indicate the safety of the compound in addition to determining its pharmacological fate following administration of this drug. This paper concerns itself with the investigation of the acute and chronic toxicity of bunamiodyl when administered orally to two species of animals. Studies dealing with the distribution and fate of bunamiodyl in animals and humans are presently being conducted and will be reported at a later date.

Toxicological studies performed in Europe (1) indicate that the LD_{50} of this material when administered intravenously to mice is 0.57 Gm. per Kg. The intravenous injection of bunamiodyl at a dose level of 0.2 Gm. per Kg. to a

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[1] Trademarked Orabilex by E. Fougera & Co., Inc.

rabbit and the intragastric administration at the same dose level to cats produced no discomfort to either species of animal. The gallbladder of the cat was opacified from the fifteenth to the sixty-fourth hour following administration of the drug (1).

EXPERIMENTAL METHODS

Acute Toxicity in Rats

The acute oral toxicity of bunamiodyl, after single administration, was determined in normal, healthy rats of the Holtzman strain, weighing 150 to 200 Gm. The test material was made up at a 10% concentration in warm distilled water and varying levels of this solution were then administered intragastrically to groups of 20 rats, on a per kilogram basis. Following the feedings, the animals were observed for a ten-day period for signs of toxicity as would be evidenced by loss of body weight, vomiting, convulsions, and/or death.

Throughout the observation period the animals were maintained on their regular diet of Fox Blox and water.

Table I indicates the level of test material and the number of rats that survived the various levels. The LD_{50} was calculated to be 2.75 Gm./Kg. of rat with a 95% confidence limit of 2.35 to 3.21 Gm./Kg. (2).

Table I.—Number of Rats Surviving Various Dose Levels

Level of Test Material, Gm./Kg.	No. of Rats	
	Fed	Dead
1.0	20	0
1.5	20	2
2.0	20	5
3.0	20	12
4.0	20	15
4.5	20	20

All of the surviving animals showed a normal gain in body weight at the end of the ten-day observation period. They ate well and showed no visual signs of any toxic effects.

Chronic Toxicity

Two species of animals, rats and dogs, were chosen for these studies. The drug was administered orally to all animals for a three-week period.

Rats.—A group of 80 normal, healthy rats of the Holtzman strain were used in this experiment. The animals were divided into four groups of 20 rats each. Three groups received the test material and the fourth group acted as controls. The test material, which dissolved upon heating, was made up in distilled water. Upon cooling, a very fine suspension resulted. The three levels administered were 100 mg./Kg., 750 mg./Kg., and 1.25 Gm./Kg. of rat. The controls received distilled water. All of the three levels were administered by gastric intubation, daily, for a three-week period.

The animals were weighed initially and weekly thereafter throughout the course of the experiment. The doses administered were changed weekly commensurate with body weight changes. Throughout the course of the experiment the animals were housed in individual cages and maintained on their regular diet of Fox Blox and water. They were carefully watched for any changes in behavior or appearance as indicated by a loss in body weight, discoloration of fur, emesis, and death.

All of the animals in the first two groups survived the experimental period of three weeks. In the third group (1.25 Gm./Kg.) three were dead one week after treatment. A weight gain was shown by each group. The mean growth curve for the four groups is shown in Fig. 1.

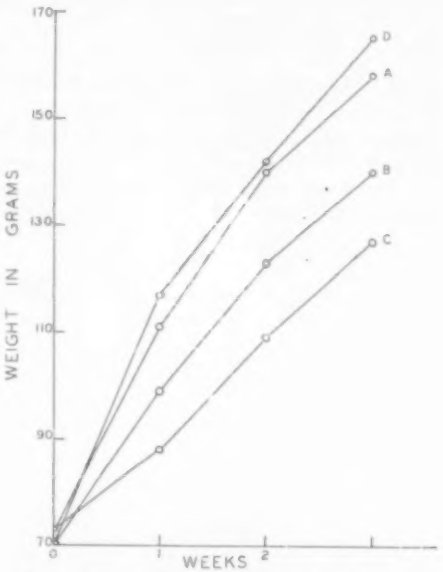

Fig. 1.—The mean growth curve of rats fed bunamiodyl at various concentrations. *A*, 100 mg./Kg.; *B*, 750 mg./Kg.; *C*, 1,250 mg./Kg.; *D*, control.

On autopsy, the macroscopic examination of all the internal organs showed a normal picture. Particular attention was given to the liver, stomach, intestines, kidney, and thyroid. The livers were normal in color and size. The stomachs were mildly distended and filled with food. The intestinal tracts were muscular and not distended. The kidneys showed no surface area of abnormality and were within the size range for this weight animal. The thyroids were normal in size and healthy in color.

Portions of the liver, kidney, intestine, and thyroid of two male and three female animals in each group were fixed in 10% formalin and prepared for histological examination. All slides were stained with hematoxylin and eosin. Microscopic examination of the prepared tissue showed a normal picture. No abnormalities were found in any of the organs examined.

Dogs.—Ten normal, healthy dogs of mixed breed were used as test animals. The animals were divided into three groups of three test animals and one control animal. Group 1 received a level of 100 mg./Kg., group 2 received 1 Gm./Kg., group 3 received 1.5 Gm./Kg. of body weight.

Upon admittance to the laboratory the dogs were placed in quarantine for a two-week period. The animals were all dewormed and received the recommended doses of canine distemper vaccine and infectious canine hepatitis vaccine.

At the end of this preliminary treatment period all the animals had a normal appetite, presented a healthy appearance, and had normal bowel elimination. They were then placed on the experimental regime. All dogs were housed in individual tile pens which were cleaned daily, and the animals were allowed to exercise in a run for an hour each day. The animals were maintained on a mixture of one part kibbled food and one part fresh cooked, ground horse meat. Fresh water was available at all times. Each animal received approximately 500 Gm. of the diet each morning. Any food that was not eaten within one hour was removed. The test material was administered on a per kilogram basis, with the material placed into No. 12 gelatin capsules. The filled capsules were placed into each of the respective dog's mouth beyond the back of the tongue and the dog forced to swallow the medication. The material was administered daily in the late afternoon for three weeks.

At the end of the experimental period the animals were sacrificed and carefully examined for any morphological changes.

Portions of the thyroid, liver, kidney, spleen, and duodenum were removed, weighed, and placed into 10% formalin solution for fixation prior to histological studies.

One of the dogs receiving 1.5 Gm./Kg. of material died on the ninth day of the experiment.

Macroscopic Examination of Organs

The livers, kidneys, spleens, thyroids, and intestinal tract of all the dogs on the 100 mg., 1 Gm., and 1.5 Gm. per Kg. of body weight were essentially normal. All the livers were clear with no adhesions present. The kidneys of each animal were normal in appearance and sections showed distinct delineation between the cortical and medullary portions. Although some of the spleens appeared to be slightly enlarged, subsequent microscopic examination showed no morphological changes. The thyroid glands of all the dogs were normal in size and appearance, and the entire intestinal tract of each dog showed no changes which could be attributed to the treatment.

Histological examination of the above organs showed no changes from untreated animals.

Liver Function Test

The cephalin flocculation test (3), which is useful in determining whether parenchymal liver damage has taken place, was performed on all dogs. The test was negative in each dog initially, and again at the end of the two-week treatment period.

Kidney Function Test

Sixteen normal, healthy dogs were used for this experiment. They were of the same type as described above and fed, examined, and housed in a similar manner. The animals were divided into three test groups and one control group. Group 1 received a level of 100 mg./Kg., group 2 received 1 Gm./Kg., and group 3 received 1.5 Gm./Kg. of body weight.

The test material was administered on a per kilogram basis, orally, each day, for a period of two weeks.

The kidney function test (4) was performed on each animal as follows: group 1 (100 mg./Kg.), initially and at the end of the experiment; group 2 (1 Gm./Kg.), group 3 (1.5 Gm./Kg.), and group 4 (controls), initially, seventh, tenth, and fourteenth day.

The test depends upon the injection of a dye, phenolsulfonphthalein, into the body tissues. This dye is eliminated rapidly and can be estimated quantitatively in the urine. It is used to indicate the amount of derangement in quantitative functional disturbance of the kidneys.

All of the experimental animals showed an elimination rate of the dye within the range obtained for the controls (Table II).

TABLE II.—TOTAL PER CENT OF PHENOLSULFONPHTHALEIN ELIMINATED

Dog No.	Initial	14 Days		
	100 mg./Kg.			
1	67	70		
2	49	57		
3	4[a]	56		
	Initial	7 Days	10 Days	14 Days
	1 Gm./Kg.			
4	77	62.5	83	50
5	73	89	76	58
6	76	78	97	62
7	75	84	65	50
8	74	62	100	43
	1.5 Gm./Kg.			
9	69	104	67	55
10	83	83	89	68
11	92	63	70	52
12	88	65	73	48
13	90	64	100	65
	Controls			
14	61	67	58	80
15	99	82	79	56
16	91	100	91	55

[a] Volume of urine obtained too low for adequate reading.

SUMMARY

When administered orally to rats, the LD_{50} of bunamiodyl was found to be 2.75 Gm./Kg.

No systemic toxicity or liver damage was found following daily administration of bunamiodyl to dogs and rats, up to 20 times the recommended human dose, for twenty-day periods. Continued administration of the test material failed to produce any histological changes in the organs of the treated animals.

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Simplified *In Vitro* Method for Evaluating the Effects of Drugs upon the Proteolytic Activity of Gastric Juice

By C. MILANI, A. FIAMENI, and A. De BERNARDI

A new *in vitro* procedure for the quantitative estimation of proteolytic activity which attempts to simulate protein digestion is described.

WHEN EVALUATING new drugs which may be administered orally, it is often necessary to determine whether the agent exerts an influence upon proteolysis within the stomach. The present work describes a new *in vitro* procedure for the quantitative estimation of proteolytic activity. This method attempts to simulate protein digestion within the stomach with subsequent measurement of amino acids released as a criterion of proteolysis. We are, therefore, able to determine the extent of normal proteolytic digestion as well as the effect of drugs upon this digestion.

The consistency of the findings enable the expression of the results numerically at various time intervals. The values obtained by this procedure have been called the Indexes of Digestion.

Gastric digestion of protein may be influenced by oral administration of drugs, which, apart from their pharmacological action, may alter the proteolytic properties of pepsin during digestion within the stomach; this may be particularly true of the antiulcer and antacid drugs.

In order to simulate actual digestive conditions within the stomach we have employed artificial gastric juice which is constantly agitated and which is continually added to the reaction vessel. This attempts to duplicate peristalsis and gastric secretion. Naimark has used the same method for different aims (1–4).

A quantitative evaluation of protein digestion may be made by various methods: i.e., determining the increase of amino acid nitrogen after Van Slyke (5–7) or after Sorensen (8); or by other procedures (9–11). These methods are generally time-consuming or not sufficiently sensitive. In this study, therefore, only colorimetric methods, which afford rapid yet accurate determinations, have been used.

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LABORATORY APPARATUS AND PRODUCTS

A 600-ml. beaker containing the artificial gastric juice, a thermostatic water bath at 38°, provided with a glass stirrer (about 7-mm. diameter) which operates at 26–28 r.p.m., and which is flexed through 90° at one end so that the horizontal part when immersed in the beaker may cover in one revolution the bottom of the beaker. A Beckman model DU spectrophotometer. Artificial gastric juice (12), prepared by dissolving 2 Gm. of sodium chloride and 3.2 Gm. of pepsin (1:3,000) in 100 ml. of distilled water and 7 ml. of concentrated hydrochloric acid; the mixture is stirred until completely dissolved, then diluted to 1 L. with distilled water; the pH of the solution is 1.2 and should be refrigerated when not in use. A 13% solution of *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid (prepared just before use). A 10% aqueous solution of trichloroacetic acid, prepared with distilled acid. Dry albumin in small pieces.

EXPERIMENTAL

Place 400 ml. and 200 ml. of artificial gastric juice, respectively, into 600 ml. beakers (A and B) placed in a thermostatic water bath at 38°.

When the juice has reached the water bath temperature add, with continuous stirring, 20 Gm. of dry albumin together with a measured quantity of substance to be examined to beaker B. After the introduction of albumin, remove at ten-minute intervals 20 ml. of the juice and add to beaker B the same aliquot of new juice taken from beaker A. Add the aliquots of juice removed from beaker B to 20 ml. of trichloroacetic acid solution and stir the mixture. Precipitation is complete after forty minutes. Filter, and from each filtrate remove 0.5 ml. of clear liquid and place into a test tube. Add to each aliquot 2.5 ml. of dilute hydrochloric acid solution and 2 ml. of *p*-dimethylaminobenzaldehyde. After careful agitation incubate the test tube at 35°. Twenty-five or thirty minutes after adding the reagent, take spectrophotometer readings at λ 542 mμ.

DISCUSSION AND EXPERIMENTAL RESULTS

The reagents applicable for this determination may be divided into two groups: those producing a color reaction with almost all the amino acids and polypeptides, and others giving a color reaction only in the presence of a specific amino acid.

We have experimented with ninhydrin (13–14), sodium β-naphthoquinone-4-sulfonate (15–16), and copper carbonate and sulfate (17–18), all belonging to the first category. None of the above-mentioned reagents were employed because of insufficient

sensitivity. We have also tried sodium nitroprusside, which falls into the second category of reagents; it is a reagent used for the determination of methionine (19–20), an amino acid which is present in abundant quantities in egg albumin (21). This reagent was not used in this study because of insufficient sensitivity.

The best results were obtained with *p*-dimethylaminobenzaldehyde, a reagent generally employed for the determination of tryptophan (22–24). The reagent gives a violet coloration in a very acid solution of tryptophan.

The absorption spectrum for tryptophan shows a maximum absorbance at λ 542 mμ. This wavelength was used for all our determinations. *p*-Dimethylaminobenzaldehyde exhibits maximum development of coloration when dissolved in concentrated hydrochloric acid. The color decreases with the lowering of the hydrochloric acid concentration. Evaluations made on a 0.1% solution of tryptophan in water (Fig. 1) indicate that with 2 ml. of 13% reagent, the maximum absorbance is obtained. The presence of aldehydes and quantities of trichloroacetic acid greater than those specified for the determination may interfere with the intensity of the color reaction.

Absorbances were determined on a solution of tryptophan at progressive time intervals. The data are shown in Fig. 2. It should be noted that the readings must be made between twenty-five and thirty minutes after adding the chromatic reagent at 35°; and between seventy and eighty-five minutes at 18°.

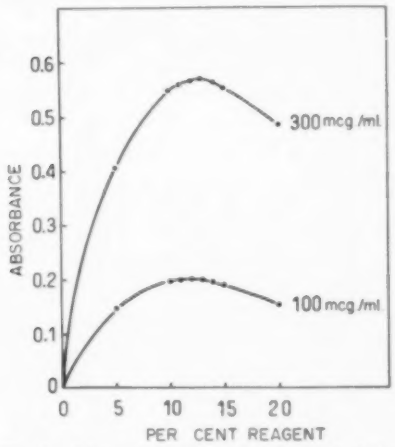

Fig. 1.—The per cent concentration effect of the reagent on absorbances, obtained on two tryptophan specimen solutions, λ 542 mμ, 10-mm. cells.

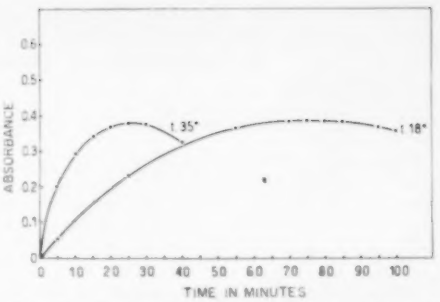

Fig. 2.—Temperature effect on the development time and on the stability of coloration. Tryptophan specimen solution 200 mcg./ml., λ 542 mμ, 10-mm. cells.

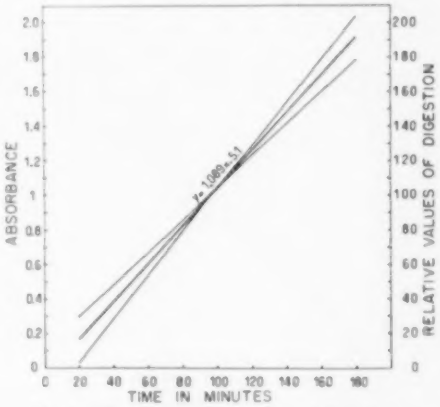

Fig. 3.—Standard curve of normal digestion.

The absorbances obtained with increasing quantities of tryptophan follow the Lambert-Beer law.

The quantity of albumin added as the substrate for digestion varies with the time of determination and the quantity of gastric juice employed.

Reproducibility of this procedure is exceedingly good as demonstrated in Fig. 3 and Table I.

The data indicate first an increase of the absorbance values, then a decrease, which gives a line parallel to the abscissa. The removal of aliquots prevents a true proportional increase which would be obtained by referring to the diagram for a sum of the absorbances. In order to equate the extent of proteolysis with time, an Index of Digestion has been formulated which may be calculated as follows

TABLE I.—STATISTICAL DATA

Sources of Variability	Degrees of Freedom	Sum of Squares	Mean of Squares	F	P
Regression	1	199.474	199.474	311.678.10	0.001 < P
Deviation from linearity	7	22	3.14	4.91	0.05 P > 0.01
Between points	8	199.496.03	24.937	3.117.10	0.001 > P
Error	54	34.67	0.64	...	...
Total	62	199.530.70	...	...	...

TABLE II.—DIGESTION INDEXES

Time	Indexes of Digestion, min. 60	120	180	Fig. 4 Curves
Normal digestion	1.00	1.04	1.05	A
+ Neomycin sulfate (1.5 Gm.)	0.80	0.94	0.94	B
+ A commercial antacid (2 tablets)	0.80	0.86	0.89	C
+ Sodium fluoride (0.5 Gm.)	0.76	0.79	0.83	D
+ Sodium bicarbonate (4.5 Gm.)	0.26	0.26	0.41	E

$$ID = \frac{\text{absorbance} \times 100}{\text{time}}$$

A comparison of the effects of several experimental compounds upon the proteolytic activity of gastric juice has been determined and data expressed in terms of absorbances in Fig. 4 and as Indexes of Digestion in Table II.

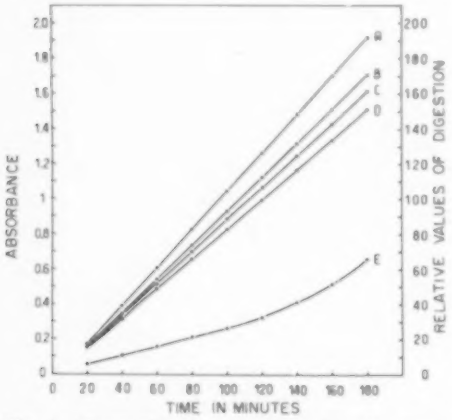

Fig. 4.—Comparison between normal peptic digestion and in the presence of some substances.

CONCLUSIONS

1. The method is applicable for estimating quantitatively *in vitro* proteolytic activity of gastric juice in the presence of drugs.

2. The Index of Digestion is a value which expresses the relationship of proteolysis *versus* time.

3. The Index of Digestion is inversely proportional to the ability of an agent to interfere with protein digestion within the stomach and directly proportional to the proteolytic capacity of the gastric juice.

4. The inhibitory effect upon normal proteolysis of the substances investigated decreases progressively with time.

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Phytochemical Investigation of a *Rubiaceae, Galium triflorum*

By ROBERT P. KNOTT and ROB S. McCUTCHEON

The phytochemical investigation of a northwest herb, *Galium triflorum*, is presented including a study of the physical and chemical properties and methods of isolation of asperuloside, a glucoside common to many members of the *Rubiaceae*. The hypotensive effects of asperuloside and an aqueous extract of the plant were tested pharmacologically. The results obtained are presented.

GALIUM TRIFLORUM is a species of small herb found widely distributed in North America (1, 2). Many medicinal claims have been made for this plant and related species, but few have been substantiated by a scientific study (3). Delas (4) has reported the most significant observation in that aqueous extracts of *Galium aparine*, a closely related species, exert a hypotensive effect when administered intravenously to dogs. It was also of interest that asperuloside has been isolated from many plants of the *Rubiaceae* (5–12) and might be found in *Galium triflorum*.

This investigation was undertaken in order to search for the presence of asperuloside and other substances of potential medicinal importance and to attempt to determine the possible medicinal value of the plant by ascertaining the proximate constituents, the vitamin concentration, the mineral content of the ash, and to test the hypotensive effects of plant extracts and principles isolated from them.

EXPERIMENTAL

Collection and Preparation of the Plant.—The samples used in this study were collected in September and early October 1958, in and around Benton County, Oregon (2).[1] The plant was air-dried under conditions of low humidity. The roots were separated from the aerial parts of the plant and the latter were ground in a Wiley mill to a number 40 powder and stored in tightly sealed cannisters at room temperature. Since the amount of root obtained was too small for study, all determinations were made on the aerial parts in duplicate, being repeated when necessary until checks were obtained.

A histological study of the pulverized plant revealed the presence of crystals and raphides of calcium oxalate, unicellular, nonglandular hairs, and stomata of the rubiaceous type. Starch grains were absent.

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Abstracted from a dissertation submitted to the Graduate School of Oregon State College by Robert P. Knott in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] The plant was verified by Dr. Albert N. Steward of the Oregon State College Herbarium.

Partial Proximate Analysis.—In the determinations that follow, methods as outlined in the "Official Methods of Analysis" (13) were used unless otherwise noted. The moisture determinations were made using the U. S. P. XV (14) gravimetric method. The total ash, water-soluble ash, and acid-insoluble ash determinations were carried out using a slight modification of the "Official Methods of Analysis" (13) method. The modification consisted of lowering the incineration temperature to 477° to prevent the loss of potassium. A polysaccharide was obtained by the official method for starch but starch was not confirmed and the substance was assumed to be mucilagenous in nature. The results are presented in Tables I and II.

TABLE I.—PARTIAL PROXIMATE ANALYSIS OF *Galium triflorum*

Substance	%
Moisture (dried plant)	8.41
Moisture (fresh plant)	82.95
Total nitrogen	1.58
Reducing sugar	1.57
Sucrose	3.44
Starch	Negative
Crude fiber[a]	21.93
Polysaccharide	10.56

[a] Expressed as per cent of the ether-soluble residue.

TABLE II.—COMPOSITION OF THE ASH OF *Galium triflorum*

Substance	%
Water-soluble ash	3.30
Acid-insoluble ash	6.63
Total ash	17.37
Aluminum oxide	1.02
Ferric oxide	0.13
Calcium oxide	2.74
Magnesium	0.42
Potassium	2.73
Sodium	Trace
Chloride	0.63

Extractions.—Samples of the aerial parts of the dried plant were extracted selectively in a Soxhlet apparatus by a continuous extraction method using petroleum ether, benzene, chloroform, alcohol, and water in the order named. The extracts were evaporated, dried to constant weight at 100°. The results are presented in Table III.

Studies of the water and alcohol-soluble extracts of the dried plant indicated the presence of a mucilagenous substance, an organic acid, and the absence of tannins and alkaloids.

TABLE III.—SELECTIVE EXTRACTION OF *Galium triflorum*

Substance	%
Petroleum ether extract	2.02
Benzene extract	2.99
Chloroform extract	0.81
Alcohol extract	11.39
Water extract	15.24

Special Extractions.—Coumarin was extracted from the dried plant by two methods and identified by melting point and mixed melting point. One procedure involved direct extraction of 21 Gm. of the dried plant by addition of 150 ml. of water and boiling for fifteen minutes. The solution was strained hot and the residue washed with two 150-ml. portions of hot water which were then combined, filtered, and allowed to cool. The aqueous extract so obtained was extracted three times with 300-ml. portions of chloroform. The chloroform extracts were combined, the chloroform evaporated on a steam bath, and the coumarin obtained by recrystallizing from boiling water. The yield was approximately 0.28% crude coumarin. In the second procedure, 450 Gm. of the dried plant was extracted with acetone in the Soxhlet apparatus. Following evaporation of the acetone the residue was extracted with boiling water (11).

Asperuloside could not be isolated from the fall-gathered, air-dried plant by employing the method of Herissey (6), Trim and Hill (12), or that of Plouvier (11).

Samples of a spring collection that were oven-dried at 49° yielded 0.12% of asperuloside following the extraction procedure of Plouvier (11). The isolated glucoside gave the following constants: m. p. 127–129°; $[\alpha]_D^{25}$ −194.2 (C 1.0, water) for the hydrous glucoside, ultraviolet absorption maximum of 238 mμ (5×10^{-2} Gm./L.) as seen in Fig. 1, and an infrared spectrum having peaks as follows: 3350s, 3000s, 2940s, 2885s, 1742s, 1700s, 1660s, 1382s, 1365s, 1340s, 1262s, 1174s, 958s, 915s, 860s, 811m, 764w, 740w, 725w, and 675w (potassium chloride pellet) shown in Fig. 2. A spectrum obtained using a Nujol mull gave similar results, illustrated in Fig. 3. These spectra as well as the infrared spectrum for the black polymer obtained by acid hydrolysis of asperuloside showed some difference from the results of Briggs and Cain (15), who investigated asperuloside isolated from members of the *Coprosma* genus. The principal difference here is the absence of the peak at 1786 for asperuloside which they had reported.

An elemental analysis of the isolated glucoside showed: carbon, 49.91%; hydrogen, 5.61%; nitrogen, halides, and sulfur absent. An acetyl determination indicated the presence of 9.04% CH_3CO or one acetyl group in the molecule of asperuloside based on a molecular weight of 410.

Aqueous solutions of asperuloside produce a green coloration upon the addition of acid which gradually turns into a greenish-black precipitate. Briggs and Cain (15) speak of this precipitate as being a polymer of toluquinhydrone. This test is fairly sensitive for asperuloside although not specific and has been used by some investigators in order to determine the possible presence of this glucoside in different members of the family *Rubiaceae*.

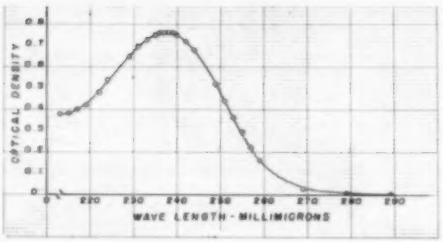

Fig. 1.—Ultraviolet spectrum of asperuloside concentration 5×10^{-2} Gm./L. in water. Readings made with a Beckman model DU spectrophotometer.

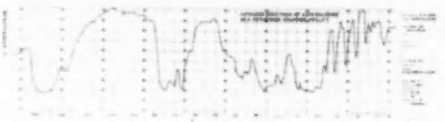

Fig. 2.—Infrared spectrum of asperuloside in a potassium chloride pellet.

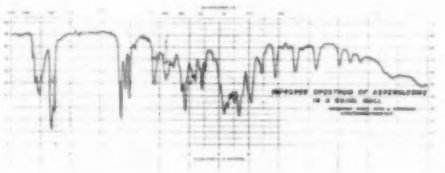

Fig. 3.—Infrared spectrum of asperuloside in a Nujol mull.

Aqueous solutions of asperuloside readily decolorize 0.1 *N* permanganate and reduce Tollens' reagent. The glucoside would not reduce Fehling's solution, gave a negative ferric chloride test, and did not decolorize bromine in carbon tetrachloride at room temperature. Because asperuloside is unstable in aqueous solution, particularly in the presence of acid or base, solutions were prepared for all determinations immediately before use. The structure proposed by Briggs and Cain for this compound is shown in Fig. 4.

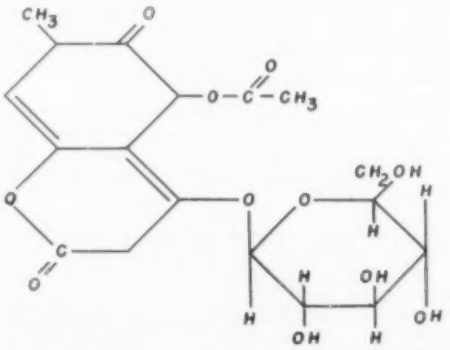

Fig. 4.—Proposed structure for asperuloside.

When the dried plant was assayed for carotene, nicotinic acid, and ascorbic acid, only carotene was found in measurable amount.

Pharmacological Tests.—Aqueous extracts of the aerial portions of the air-dried fall collection of *Galium triflorum* produced a fall up to 30 mm. in the blood pressure of dogs when given intravenously. A similar response was noted from extracts that were freed of coumarin and the sugars present in the plant. Asperuloside in a dose of 4 mg./Kg. lowered blood pressure of the rabbit an average of 12 mm. When the dose was doubled the average blood pressure fall was 27 mm. In all cases the animal showed a rapid but incomplete recovery from the depressor effect. Asperuloside failed to lower the blood pressure of dogs at a similar dosage. Preliminary acute toxicity studies indicated that asperuloside showed no deleterious effects on mice when given intraperitoneally in doses ranging from 50 to 400 mg./Kg.

DISCUSSION

The nitrogen content of the plant was lower than that normally found. The fact that the material used for this determination was the aerial parts of the fall-gathered plant may partially explain this result. The sugar and crude fiber content were found to be high. The high crude fiber could possibly be explained by the fact that the plant was very stemmy.

Asperuloside could only be extracted from the aerial parts of the spring-gathered plant but not from the fall-gathered plant, indicating that the plant content of this glucoside varies with the season and that the fresh young plant contains the highest percentage of asperuloside. Table IV, which compares yields obtained from species of *Galium* or closely related genera, tends to support this consideration. Trim and Hill (16) have stated that the asperuloside content of *Galium aparine* increases as the plant matures and then begins a period of glucoside depletion.

TABLE IV.—YIELDS OF ASPERULOSIDE OBTAINED FROM DIFFERENT PLANT SOURCES

Source	Part Used	% Yield
Galium verum (9)	Dried aerial parts	0.05
Asperula odorata (6)	Dried aerial parts	0.05
Rubia tinctorum (12)	Fresh young root tops	0.58
Galium aparine (12)	Dried seedlings	0.05
Galium aparine (12)	Fresh young root tops	1.08
Galium triflorum	Dried aerial parts	0.12

The fact that Briggs and Nicholls (5) obtained 2.9% asperuloside from the air-dried bark of *Coprosma tenuifolia* and Plouvier (11) has reported still higher yields from the dried leaves of *Escallonia* would indicate a considerable variation in the asperuloside content in species of the different genera from which this glucoside has been isolated.

A study of the chemical and physical properties of asperuloside showed them to be similar to those reported by other investigators. Ultraviolet and infrared data suggest that the asperuloside of *Galium triflorum* is a highly unsaturated compound generally in agreement with the proposed structure of Briggs and Cain (15). Asperuloside is believed to be the beta-glucoside of an enolic lactone. Enough differences in the physical and chemical properties exist to warrant consideration of the possibility that the *Rubiaceae* family contains more than one glucoside of what may be called the asperuloside type.

Aqueous solution of the dried aerial parts of the fall-gathered collection, from which no asperuloside could be isolated, were shown to produce a depressor effect on the blood pressure of dogs. The effect of asperuloside on the bood pressure was not definitely established but indications are that it is not the principal constituent of the plant responsible for the depressor effect.

SUMMARY

1. A partial proximate analysis and the mineral content of *Galium triflorum* has been completed.

2. A histological study of the pulverized plant has been carried out.

3. A selective extraction of the plant showed high content of alcoholic and aqueous extracts.

4. Coumarin and asperuloside were isolated from the plant.

5. The physical and chemical properties of asperuloside were studied and compared with data reported by Briggs and other investigators.

6. Preliminary pharmacological tests of effects of extracts as well as asperuloside on blood pressure were made.

7. Acute toxicity of asperuloside was tested in mice.

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Pfizer Tablet Hardness Tester

By H. J. FAIRCHILD and F. MICHEL

Hardness has long been regarded as an important quality characteristic of tablets. Until recently, only two practical types of tablet hardness testers were available to the pharmaceutical industry. A new addition to this field of testing has just been made. An evaluation of the new instrument includes test comparisons against existing tablet hardness test equipment.

Hardness is the physical property of compressed dosage forms which is a measure of resistance to chipping, abrasion, or breakage under conditions of storage, transportation, and handling before usage (1). Generally, the ability to withstand handling, packing, and shipping has been associated with the amount of strength possessed by tablets. This may be tested by breaking the tablet between the second and third fingers when using the thumb as a fulcrum, thereby producing a sharp snap; also, shaking well a few tablets in cupped hands to simulate shipping (2).

The above test procedure is called an "arbitrary 'rule of thumb' method" (3). It is almost a useless method, especially when the desired hardness is so high that the tablet is virtually impossible to break by the described procedure. Since hardness is one of the important factors which must be correlated with disintegrating characteristics of tablets (4), it is logical that mechanical hardness testers should have been developed to replace the "rule of thumb" method and establish an objective measure of this important quality characteristic.

HISTORICAL

The first widely acceptable tablet hardness tester, made by Monsanto, was introduced in the mid 30's (5). The Monsanto hardness tester (now distributed by Stokes) measures the diametrically applied force which results in tablet breakage. The force is generated by the compression of a coil spring (Fig. 1). The instrument has attained the status of a standard tool of quality control in the manufacture of compressed tablets. The value of the instrument lies in its ability to reproduce a test over a wide range of forces, up to a maximum of 20 Kg. The instrument is small, portable and fairly easy to use. Results can be read to the nearest 0.5 Kg.

A second instrument was developed fifteen years later by Robert Albrecht (6) and eventually distributed as the Strong Cobb tablet hardness tester (Fig. 2). This unit is also intended to measure the diametrically applied force required to break a tablet. The force is produced by a manually operated air pump. As the pressure increases, a plunger is forced against a tablet set on an anvil. A dial indicator records the final breaking force. The dial is calibrated to thirty units of force. These units, however, are neither representative of kilograms nor pounds. A study by McCallum, *et al.* (7), shows that each unit of calibration is approximately 0.6 Kg. This instrument is large and requires some bench or table space in order to perform tests.

Fig. 1.—Stokes (formerly Monsanto) tablet hardness tester.

In 1956, Shafer, *et al.* (8), reported on the development of the Roche friabilator. They held that resistance to compression is not, as a rule, a serious factor in the life of most tablets. Instead, they held that abrasion and shock are the forces which most often cause tablets to chip or break. The unit, illustrated in Fig. 3, induces self abrasion of the tablets as the cylinder section rotates. Shock results as the tablets fall six inches on each turn. After four minutes of this treatment, the tablets are weighed and compared to their initial weight. The per cent of weight lost due to dusting is the measure of tablet friability. Acceptable limits of weight loss were based upon correlation with actual cross-country shipments as well as local truck deliveries.

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Fig. 2.—Strong Cobb tablet hardness tester.

Fig. 3.—Friabilator made to Roche specifications.

Each of the above mentioned devices has been used to control and evaluate the hardness or friability characteristics of tablets produced by Pfizer. The Stokes and Strong Cobb hardness testers, or any similar devices, measure only those qualities related to tablet resistance to crushing (4). Abrasion and shock effects can be measured to some degree by the Roche friabilator, or to a greater degree by subjecting packaged material to recommended National Safe Transit Committee tests (9). The latter are intended to simulate actual handling and shipping vibration, as well as shock or impact to packaged products, and the test conditions are controllable, reproducible, and correlated with normal shipping conditions.

Hardness has been associated with other tablet properties such as density and porosity, which usually affect disintegration rates (4, 10). Hardness has generally shown a tendency to increase with the aging or storage of tablets (3, 11). Tablets should not be harder than is necessary for adequate handling and shipping. This degree of hardness can be predetermined and controlled. A new tool for the accomplishment of this purpose is the Pfizer tablet hardness tester (12).

This instrument was designed to make hardness testing easier, faster, and more reliable. The instrument is light in weight and can be used in the laboratory or at the tablet making machine. It is small enough to fit an operator's pocket. It has dial readings in both pounds and kilograms, calibrated to 0.5 lb. and 0.2 Kg., respectively. The safe testing capacity of the instrument is 35 lb.

EXPERIMENTAL

Design.—The Pfizer hardness tester utilizes the mechanical principle of ordinary pliers. As the tablet is crushed in the jaws of the device, the force is recorded on a dial indicator (Fig. 4*b*). A schematic diagram of the instrument is shown in Fig. 4*a*. The dial (*A*) has a single indicator which remains at the tablet breaking reading after each test. The

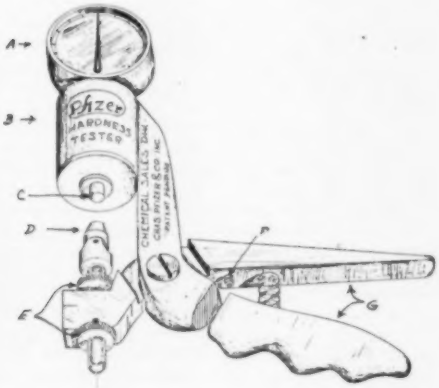

Fig. 4*a*.—Pfizer tablet hardness tester, schematic of pilot model.

Fig. 4*b*.—Pfizer tablet hardness tester, actual production model.

TABLE I.—HARDNESS DETERMINATIONS ON "STANDARD" TABLETS

Tablets	Tester	Mean, Kg.	Standard Deviation	Coefficient of Variation, %
Soft	Pfizer	4.1	0.35	9
Soft	Monsanto	4.4	0.6	13
Soft	Strong Cobb	6.4[a]	0.95	15
Hard	Pfizer	7.4	0.84	11
Hard	Monsanto	7.5	0.75	10
Hard	Strong Cobb	12.4[a]	1.47	12

[a] These are arbitrary units, not standardized to either pounds or kilograms.

TABLE II.—COMPARATIVE HARDNESS OF FIVE TABLET PRODUCTS

Tablet	Tester	Mean	Standard Deviation	Coefficient of Variation, %
Cold tablet	Pfizer	11.0 lb.	1.2 lb.	10.9
Cold tablet	Monsanto	4.7 Kg. (10.4 lb.)	0.63 Kg.	13.4
Aspirin	Pfizer	12.2 lb.	1.5 lbs.	12.0
Aspirin	Monsanto	5.7 Kg. (12.5 lb.)	0.82 Kg.	15.0
Appetite builder	Pfizer	20.2 lb.	1.76 lb.	8.7
Appetite builder	Monsanto	9.5 Kg. (21.0 lb.)	1.12 Kg.	11.5
Penicillin	Pfizer	17.4 lb.	3.1 lb.	18.0
Penicillin	Monsanto	8.5 Kg. (18.7 lb.)	1.6 Kg.	19.0
Aspirin (child's)	Pfizer	8.6 lb.	1.18 lb.	13.7
Aspirin (child's)	Monsanto	3.3 Kg. (7.2 lb.)	0.38 Kg.	11.5

indicator returns to zero when a reset button on top of the dial is depressed. The spring housing (*B*) affords dust and moisture protection to the spring as well as the mechanism. The plunger (*C*) compresses the spring during the hardness determination. The tablet is placed on edge on the self-aligning swivel stage (*D*) which can be hand-adjusted to accommodate various tablet sizes by means of knurled lock nuts (*E*). Adjustments need not be made for small differences in tablet sizes. An adjustable stop screw (*F*) arrests the lower handle and prevents the swivel stage from contacting the dial-activating plunger after a tablet breaks. The hand grips (*G*) are designed for maximum comfort and ease of operation. The contoured lower lever assures nonslip performance during testing.

Ease of Operation.—The first pilot model of the Pfizer tester was subjected to many types of evaluations. Inexperienced personnel, for example, were handed the instrument and a tablet and asked to test the tablet on end and report the hardness. Each was able to perform the test quickly without further instruction and report a correct result. The same experiment, repeated on other inexperienced persons but using the Monsanto or the Strong Cobb testers, required considerably more time and, sometimes, additional instruction before a successful test could be performed.

Comparison of Accuracy and Reliability Using Standard Tablets.—The most important evaluation of the instrument sought to determine its accuracy and reliability. To do this, some standard tablets were made. A granulation was screened to a particular particle size and tableted on a single punch machine at two different pressure settings. The tablets produced at each pressure setting were tested on the Pfizer, Monsanto, and Strong Cobb testers, respectively. Samples of 25 tablets were used on each instrument and the mean, the standard deviation, and the coefficient of variation, which is the standard deviation expressed as a per cent of the mean, were computed for each sample. The results are shown in Table I.

The values on the Pfizer and Monsanto testers checked each other fairly well. The Strong Cobb readings were approximately 1.4 to 1.7 times the absolute values recorded on the other instruments. The values for the coefficients of variation were of the same order and, for this experiment, indicate that even in early models, the reliability of the Pfizer tester was at least as good as that of the others.

Comparison of Accuracy and Reliability Using Tablets of Various Manufacturers.—A series of comparisons was made between the Pfizer and Monsanto testers only. Tablets of various manufacturers were tested as before and produced the results shown in Table II. The Monsanto tester readings show a conversion of Kg. to lb., in parentheses, for ease of comparison of results obtained with the Pfizer tester. The five products tested provide a fair range of tablet hardnesses but are not necessarily representative of all tablets produced by the pharmaceutical industry.

The results in Table II further established the reliability and accuracy of the Pfizer tester. The values obtained for the coefficient of variation also show the wide variation that exists in tablet hardness, regardless of the product. A verification of this observation was made by analyzing the results reported for nine tablet products studied by McCallum, *et al.* (7), when they compared the Monsanto and Strong Cobb tablet hardness testers. For each set of data in Tables I, II, and III of their report, the standard deviations were computed. These are presented in Table III as the coefficients of variation. The mean or average hardnesses are included in this table for their comparative informational value only.

TABLE III.—COMPARISON OF MEAN HARDNESS AND STANDARD DEVIATION ON STRONG COBB AND MONSANTO TABLET HARDNESS TESTERS[a]

Tablet	Strong Cobb		Monsanto, Kg.	
	Mean	Coefficient of Variation, %	Mean	Coefficient of Variation, %
Sodium bicarbonate	16.9	4.8	10.1	8.0
Ferrous sulfate	12.1	7.0	7.4	8.9
Sodium salicylate	12.1	11.1	7.9	14.2
Acetylcarbromal	6.1	11.0	3.6	15.6
Digitalis	4.0	5.3	2.25	10.2
Calcium carbonate, magnesium oxide	14.3	7.3	9.9	9.7
Ferrous sulfate, quinine sulfate	12.5	14.8	7.4	19.0
Ferrous sulfate, cobalt sulfate	11.3	6.7	7.3	12.5
Aspirin compound	11.3	13.8	7.4	19.5

[a] Data in this table compiled from test results appearing in ref. (7).

The average of the coefficient of variation obtained on Strong Cobb readings is about 9% as compared to 13.5% on Monsanto readings. Most of this variation is due to the tablets, but some can be attributed to the instrument or the operator technique. This was demonstrated by testing tablets on the same instrument while varying the rate of pressure increase. A Monsanto tester was used. For the first sample, the threaded shaft was turned slowly; for the second, as fast as possible. The results are shown in Table IV.

TABLE IV.—COMPARISON OF SLOW AND FAST TESTING ON THE SAME MONSANTO HARDNESS TESTER

Kg.	Slow Test	Fast Test
6.0	X X X X	X X
5.5	X X X X X X X X X	X X X
5.0	X X X X X X X X	X X X X X X X X X
4.5	...	X X X X X
4.0	X	X X
	Average, 5.6 Kg. Standard deviation, 0.48 Coefficient of variation, 8.5%	Average, 5.2 Kg. Standard deviation, 0.55 Coefficient of variation, 10.6%

Fig. 5.—Pfizer tablet hardness tester in use.

The last major observations made during the evaluation of the Pfizer hardness tester concerned its speed of operation. A tablet hardness determination can be obtained four to seven times faster than on the Strong Cobb or Monsanto testers. The reason for this is obvious (Fig. 5). The Monsanto tester requires a series of turns of the screw to generate the pressure required to break the tablet. An equal number of reverse turns is required to prepare the instrument for the next test. The Strong Cobb tester requires a continuous pumping action to generate the air pressure which activates the plunger. The Pfizer tester, on the other hand, requires a single, squeezing action to generate the necessary force. Release of the hand pressure opens the jaws of the instrument immediately. Reading, recording, resetting, and cleaning, if necessary, can be accomplished almost instantaneously.

SUMMARY

1. Tablet hardness is an important quality characteristic because of its relationship to many other tablet properties.

2. The Pfizer tablet hardness tester provides an improved method for determining tablet hardness, offering ease and speed of performance as well as accuracy and reliability of results.

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Preliminary Studies on Use of Sensitized Guinea Pigs in the Evaluation of Protective Ointments

By JOY BICKMORE PLEIN, ELMER M. PLEIN, and JACK A. NASH

A procedure for the development of allergic contact dermatitis in guinea pigs in which six potential allergens were used and a method for testing the effectiveness of barrier ointments, creams, and aerosols in preventing contact dermatitis in sensitized guinea pigs (to oster oil) have been described.

THE CLINICAL testing and screening of protective ointments, creams, and aerosols presents the obvious difficulty of finding a sufficient number of sensitized test subjects and also presents the danger of exposing a sensitized individual to allergen. Eisen and his co-workers and a number of other investigators have described the production of allergic dermatitis in guinea pigs by the topical application of dinitrobenzene compounds (1–7), and Graul and Kalkoff (8) published a report of the sensitization of guinea pigs to topical application of inorganic salts. It was felt that a procedure involving application of test allergens to the ointment-protected skin of dermatologically sensitized guinea pigs might prove an effective method of evaluating protective ointments.

EXPERIMENTAL

Young female guinea pigs weighing about 350 Gm. at the beginning of the study were used as the experimental animals. The hair of the dorsal skin was clipped with a Raycine electric clipper.

Sensitization of Guinea Pigs.—Development of sensitization to the following potential allergens was attempted: dinitrofluorobenzene (0.01 *M* in alcohol-propylene glycol solution and in acetone-corn oil solution), ethyl aminobenzoate (5% in white petrolatum), sodium lauryl sulfate (5% aqueous solution), procaine hydrochloride (2% aqueous solution), chlorpromazine (2.5% aqueous solution), and oster oil (a cutting oil used by machinists).

Three procedures were studied for the sensitization of guinea pigs to dinitrofluorobenzene (DNFB). None of these methods employed the use of defatting solutions prior to the application of the potential allergen. In the first series, 0.07 ml. (3 drops) of 0.01 *M* 1-fluoro-2,4-dinitrobenzene (Eastman) in absolute alcohol-propylene glycol, 1:1 solution, was applied to the anterior dorsal skin of each of ten guinea pigs of assorted skin colors. The solution was rubbed in, allowed to remain on the skin for 10 minutes, and was then removed with cleansing tissues. Application was repeated daily for a total of seven doses. The guinea pigs were allowed to rest 10 days and then an 0.045-ml. test dose was applied. The test dose showed low sensitization (see results) to DNFB in this vehicle and, therefore, as soon as the reactions cleared (2 days), these same guinea pigs were treated with 0.07 ml. of 0.01 *M* DNFB solution in acetone-corn oil 1:1. The solution was rubbed in and allowed to remain on the skin rather than being removed 10 minutes after application. Treatment was continued each day for 7 days and, after a 7-day rest period,[1] an 0.05-ml. test dose was applied. Five albino guinea pigs were used in a second experiment with DNFB in acetone-corn oil 1:1. In this series, the solution was merely dropped on the skin rather than applied with rubbing because it had been found that this procedure seemed to result in a more intense reaction. Application was repeated each day for 7 days and, after a 10-day rest period, an 0.07-ml. test dose was applied. With these five animals, the vehicle was applied to another area to test for reaction to the acetone-corn oil solution. Throughout the study the extent of the skin reaction at about 24 hours following the application of the allergen was recorded. These reactions were read by at least two investigators and evaluated according to the severity of the reaction: 0, no reaction; 1+, slight erythema; 2+, erythema; 3+, marked erythema; 4+, severe erythema with swelling.

In attempting to sensitize the animals to application of ethyl aminobenzoate, sodium lauryl sulfate, procaine hydrochloride, chlorpromazine, and oster oil, the skin was defatted by cleansing it three times with a cotton swab which had been soaked in a solution of acetone-alcohol 1:1 (a less potent defatting solution was used for a portion of the first applications) prior to the application of the potential allergens. The dorsal skin was divided into five areas and as many as four substances were applied to the same animal on the same day, with the fifth area serving as a control. Potential allergens were allowed to remain on the skin for 10 minutes and then removed with cleansing tissue. Application was made at weekly intervals for 3 weeks. Initially, an amount of the allergenic preparation sufficient to cover an area about 1 inch × 1 inch was used. In later series with oster oil, 0.03 ml. (1 drop) was used. Reactions were evaluated about 24 hours after application of the allergen using the same criteria as was used in the DNFB series. Eleven to 17 animals were used in the testing of each potential allergen.

Evaluation of Barrier Preparations against Oster Oil.—The ability of eight barrier ointments and creams and one aerosol to prevent the contact dermatitis resulting from application of oster oil to

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[1] In some cases a 7-day period is sufficient for the sensitization reaction to clear and in others as much as 10 days must elapse before the skin is normal and a test dose can be applied.

TABLE I.—REACTIONS TO INITIAL AND TEST DOSES OF POTENTIAL ALLERGENS

Allergen	No. of Guinea Pigs	Reaction[a] to First Dose: No. of Guinea Pigs	Reaction[a] to First Dose: Response	Reaction[a] to Test Dose: No. of Guinea Pigs	Reaction[a] to Test Dose: Response
0.01 *M* DNFB in alcohol-propylene glycol	9	9	0	6	0
				3	1+
0.01 *M* DNFB in acetone-corn oil	9	3	0	3	1+
	(From expt. above)	2	1+	4	2+
		1	2+	2	3+
		2	3+	..	..
		1	4+	..	..
	5	1	0	5	3+
	(Albinos not previously treated with DNFB)	4	1+	..	..
5% Sodium lauryl sulfate	11[b]	10	0	2	0
		1	1+	1	?1+
				8	1+
	6	4	0	[c]	[c]
	(Albinos)	2	1+	[c]	[c]
5% Benzocaine	11[b]	10	0	4	0
		1	1+	1	?1+
				6	1+
3% Procaine hydrochloride	10	7	0	10	0
		2	?1+	..	..
		1	1+	..	..
2.5% Chlorpromazine	11[b]	11	0	11	0
Oster oil	11[b]	3	0	3	1+
		4	?1+	7	3+
		4	1+	1	4+
	6	4	1+	5	2+
	(Albinos)	2	2+	1	3+

[a] 0, No reaction; ?1+, questionable slight erythema; 1+, slight erythema; 2+, erythema; 3+, pronounced erythema 4+, intense erythema with swelling.
[b] A less potent defatting solution was used prior to the initial application than prior to second and test applications of the allergen.
[c] Test not conducted.

sensitized guinea pigs was determined. The protectants studied were white petrolatum, Kerodex[2] 51, Covicone,[2] Silicote[2] ointment, Silicote[2] aerosol, Pro-derna,[2] Vanisil (9, 10), Van Pet (10), and hydrophilic ointment U.S.P. Either 15 or 16 guinea pigs were used to test each barrier.

After the dorsal hair had been clipped and the skin defatted three times, the area was divided into quadrants. Three different barriers were tested at one time on each animal and the fourth quadrant served as a control. An 0.5-Gm. dose (an excess) of the barrier cream or ointment was applied in the area of one quadrant for a 1-minute period, left undisturbed for 1 minute, and then the excess was removed. Application of barriers two and three to their respective quadrants followed, and then a second dose was applied in the same manner to each area. There was no treatment of the control quadrant other than use of the defatting solution. One drop of oster oil (0.03 ml.) was then placed in the center of each of the four quadrants, allowed to remain ten minutes, and then removed, with care being taken not to spread the oster oil. After a 24-hour period, the reactions were read and evaluated as described under the sensitization procedure.

RESULTS

Results of sensitization procedures to the various potential allergens are shown in Table I. As can be seen in the table, alcohol-propylene glycol did not prove to be an effective vehicle for DNFB since only three of the nine guinea pigs appeared to develop sensitivity to the drug in this medium and these reactions were only 1+ in intensity. However, the initial reaction of these same guinea pigs to the allergen dissolved in corn oil-acetone showed that either this concentration in the particular solvent was irritating to a number of animals or that they had become sensitized by the allergen when applied in the previous base.[3] The values of the test dose in the first nine guinea pigs are those which were obtained by rubbing the allergen into the skin, as was done with each dose in the sensitization process with these animals. Reactions seemed to be more intense when the allergen was applied without rubbing than when it was rubbed in; however, the difference was not statistically significant.[4] Rubbing the allergen into the skin speeded the response. In order to follow development of sensitivity to DNFB without the complication of probable sensitivity of some of the animals, the five albino guinea pigs which had not had previous exposure to DNFB were sensitized. The fact that four of the five guinea pigs showed 1+ reactions on first application indicates that 0.01 *M* concentration in acetone-corn oil is slightly irritating as well as sensitizing. There was no reaction to the acetone-corn oil solvent in the five animals tested.

Sodium lauryl sulfate and benzocaine both showed slight irritant properties in a low percentage of guinea pigs and both produced 1+ reactions to the test dose in more than half of the animals. No

[2] Proprietary topical protectives.

[3] It takes 5 or 6 days for sufficient DNFB antibody to be developed to give a skin reaction to the test dose. So the reaction shown by six of the animals to the first dose could have been either primary irritation or sensitization. It is very unlikely that the 2+, 3+, and 4+ reactions were a result of primary irritation.

[4] Throughout the study differences in the means of two groups of data were analyzed statistically by use of the Student *t*-test. Differences in which $P = 0.05$ or $P < 0.05$ were considered significant.

TABLE II—REACTIONS TO TOPICAL APPLICATION OF OSTER OIL APPLIED TO BARRIER-PROTECTED SKIN OF GUINEA PIGS

Guinea Pigs of Mixed Colors

Guinea Pig No.	Reaction[a] Through: White Petrolatum (I)	Kerodex 51(I)	Covicone (I)	Control I[b]	Silicote Ointment (II)	Silicote Aerosol (II)	Proderma (II)	Control II[b]	Vanisil (III)	Hydrophilic Ointment, U.S.P. (III)	Van Pet (III)	Control III[b]
2	3+	3+	2+	3+	3+	3+	3+	3+	3+	2+	2+	2+
3	1+	1+	1+	0	2+	2+	1+	1+	2+	2+	2+	2+
4	1+	2+	2+	1+	3+	3+	1+	1+	3+	2+	3+	3+
5	3+	3+	3+	3+	4+	3+	3+	3+	4+	3+	4+	4+
6	2+	2+	3+	2+	3+	2+	1+	1+	3+	2+	2+	2+
7	2+	2+	2+	2+	2+	2+	2+	2+	3+	3+	3+	1+
8	2+	1+	2+	2+	2+	2+	1+	2+	1+	1+	1+	0
10	1+	1+	2+	2+	2+	2+	2+	2+	2+	1+	2+	2+
11	2+	1+	2+	2+	3+	2+	2+	2+	2+	1+	1+	1+
12	2+	1+	2+	3+	3+	3+	1+	3+	3+	1+	2+	1+
Average	1.9+	1.7+	2.1+	2.0+	2.7+	2.4+	1.7+	2.0+	2.6+	1.8+	2.2+	1.8+

Albino Guinea Pigs

Guinea Pig No.	Reaction[a] Through: White Petrolatum (I)	Kerodex 51(III)	Covicone (III)	Control I[b]	Silicote Ointment (II)	Silicote Aerosol (III)	Proderma (II)	Control II[b]	Vanisil (I)	Hydrophilic Ointment U.S.P (II)	Van Pet (I)	Control III[b]
13	2+	2+	2+	2+	3+	2+	2+	3+	2+	2+	2+	3+
14	3+	4+	3+	4+	4+	3+	3+	3+	4+	3+	4+	4+
15	2+	2+	3+	3+	3+	2+	2+	3+	3+	1+	3+	2+
16	1+	2+	3+	3+	3+	2+	2+	2+	3+	2+	3+	2+
17	1+	2+	3+	2+	3+	2+	2+	3+	2+	2+	2+	3+
18	2+	[c]	[c]	2+	3+	[c]	2+	2+	2+	2+	2+	[c]
Average	1.8+	2.4+	2.8+	2.7+	3.2+	2.2+	2.2+	2.7+	2.7+	2.0+	2.7+	2.8+

[a] 0, No reaction; 1+, slight erythema; 2+, erythema; 3+, pronounced erythema; 4+, intense erythema with swelling.
[b] Control was application to unprotected skin. Numbers I, II, and III in parentheses following barriers indicate test was in conjunction with Controls I, II, and III, respectively.
[c] Test not conducted.

sensitization reactions to procaine hydrochloride or to chlorpromazine were obtained.

Oster oil was irritant as shown by a number of mild reactions on first application and it also proved to be an effective sensitizer. In most cases the reaction to oster oil became progressively more intense with each application. With the 17 guinea pigs, 13 had a more intense reaction to the second than to the first dose, and 14 had a more intense reaction to the third than to the first dose. Ten animals had a more intense reaction to the third than to the second dose, one was less intense to the third than to the second dose, and six guinea pigs had the same reaction to the second and third doses. It would appear that at least 14 of the 17 animals were sensitized and in the remaining three guinea pigs, the reactions to the sensitization procedure were probably essentially primary irritation. However, two of these three animals, when being subsequently tested with barrier ointments, developed greater reactions to oster oil than they had shown in the sensitization procedures, (with the third guinea pig the reactions were inconsistently greater) so probably all 17 guinea pigs were sensitized.

The results of tests for effectiveness of various barriers in preventing contact dermatitis resulting from oster oil application to sensitized guinea pigs are reported in Table II. It may be seen that in most instances the albino guinea pigs reacted more strongly to oster oil than did the guinea pigs of mixed colors. In comparing statistically the reaction data through each protectant with the appropriate control, it was found that for the guinea pigs of mixed colors or for the entire group of animals, the reaction through Silicote ointment was more intense than was the control reaction. The differences between the results with application of other protectants and with the unmedicated controls were not significant. With the albino guinea pigs (Nos. 13–18), there were no significant differences between the reactions through the protectants and the control reactions. There were some significant differences between protectants which allowed intense reactions and those which seemed to prevent the dermatitis. In comparing data from all guinea pigs, it was found that the oster oil reaction through Silicote ointment was significantly greater than through any other protectant except Vanisil. The reaction through Vanisil was significantly greater than that through white petrolatum, Kerodex 51, Pro-derma, or hydrophilic ointment; but differences between Vanisil and the other protectants were statistically insignificant. Van Pet gave significantly less protection against oster oil than did white petrolatum, Pro-derma, or hydrophilic ointment. Besides allowing an intense reaction, some of the

barrier preparations seemed to spread the oster oil. In these instances, the reaction extended over the area covered by the barrier rather than being confined to the site of the oster oil application.

DISCUSSION

The procedure of using sensitized guinea pigs to test barrier protection against allergic contact dermatitis depends upon producing a pronounced and predictable contact dermatitis in response to exposure to the allergen. Oster oil proved to be a fairly satisfactory oil-soluble allergen. DNFB is a potent oil-soluble allergen, but its use is limited to application areas where the animal cannot ingest the substance and there would be difficulty in testing more than one barrier per animal at any one time. None of the water-soluble potential allergens tested thus far has produced a satisfactory response and further work must be done before this method can be used to test barrier protection against water-soluble substances. Four of these barriers had been tested previously by an *in vitro* procedure involving solubility in oster oil (10). The *in vitro* results were in agreement with these *in vivo* data.

Reactions are much more intense if a defatting solution is used prior to application of the oster oil than if no defatting solution is used ($P < 0.001$), and with all allergens, the hair should be clipped before each application. The animals varied one from another in response to each allergen. Guinea pig No. 5 reacted very strongly to oster oil and responded only moderately to DNFB, whereas guinea pig No. 3 showed low sensitivity to oster oil and intense reactions to DNFB. In some instances the reaction was more severe 48 hours following application of the allergen than at the 24-hour period and further investigation will include time-reaction intensity studies. Unless the investigator uses a large group of animals, testing is necessarily slow because of the time necessary for the skin to clear after each positive reaction. The procedure could be improved with development of a more objective method of reading reactions.

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Hypocholesteremic Agents I. (*p*-Acetamidophenoxy)acetic Acid Derivatives

By SEYMOUR L. SHAPIRO, HAROLD SOLOWAY, HARRIS SHAPIRO, and LOUIS FREEDMAN

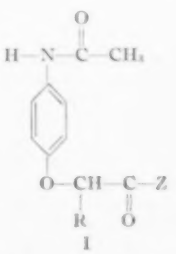
A series of (*p*-acetamidophenoxy)acetic acid derivatives (I) has been examined for hypocholesteremic effects, and significant activity was noted with R as lower alkyl and Z as an amide function.

IT HAS BEEN proposed that the hypercholesteremic state is stress-mediated (1). Since acetophenetidin purportedly influences adrenal activity via its effect on the pituitary (2) derivatives (I) of this compound have been examined, particularly for the influence on serum cholesterol levels.

Related systems have been assessed as hypocholesteremic agents (3) and for other pharmacological effects (4, 5).

For the preparation of the compounds, *p*-hydroxyacetanilide was reacted with the α-bromoester, of the formula $RHBrCCOOC_2H_5$, in acetone under reflux, employing potassium carbonate as an acid binder, to give I, Z = $—OC_2H_5$. Ammonolysis of the esters afforded the amides, and hydrolysis yielded the acids.

The hypocholesteremic effect was evaluated in guinea pigs given 30 mg./Kg. subcutaneous doses of the test compounds at the beginning of the experiment, twenty-four hours later, and finally, forty-eight hours later. Serum cholesterol levels were established at the initiation of the experiment and at the following time intervals thereafter: six, forty-eight, and seventy-two hours. The reduction (in



I

Z = —OH, $—OC_2H_5$
$—NH_2$, $—NHC_2H_5$
$—NH(CH_2)_2N(C_2H_5)_2$
$—NH(CH_2)_2OH$
R = H, $CH_3—$, $C_2H_5—$

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TABLE I.—(*p*-ACETAMIDOPHENOXY)ACETIC ACID DERIVATIVES (I)

Compound No.	Z	M. P., °C.[a]	R. S.[b]	Yield,[c] %	Formula	Analyses,[d] % Carbon—Calcd.	Carbon—Found	Hydrogen—Calcd.	Hydrogen—Found	Nitrogen—Calcd.	Nitrogen—Found
	(R = H)										
1[e]	—OH	175–177	A	32	$C_{10}H_{11}NO_4$	57.4	57.4	5.3	5.1	6.7	6.8
2[f]	$—OC_2H_5$	104–105	A	53	$C_{12}H_{15}NO_4$	60.8	60.2	6.4	6.0	5.9	6.3
3[g]	$—NH_2$	197–199	A	56	$C_{10}H_{12}N_2O_3$	57.7	57.4	5.8	5.8	13.5	13.5
4	$—NHC_2H_5$	202–203	A	39	$C_{12}H_{16}N_2O_3$	61.0	61.3	6.8	6.9	11.9	12.0
5	$—NH(CH_2)_2N(C_2H_5)_2$	73–75	B	27	$C_{16}H_{25}N_3O_3$	62.5	62.8	8.2	7.9	13.7	13.8
	(R = CH_3—)										
6	—OH	173–174	A	51	$C_{11}H_{13}NO_4$	59.2	59.1	5.9	5.7	6.3	6.4
7	$—OC_2H_5$	[h]		61	$C_{13}H_{17}NO_4$	62.1	62.4	6.8	7.0	5.6	5.7
8	$—NH_2$	200–202	C	52	$C_{11}H_{14}N_2O_3$	59.5	59.1	6.4	6.1	12.6	12.6
9	$—NHC_2H_5$	164–167	A	33	$C_{13}H_{18}N_2O_3$	62.4	62.3	7.3	7.1	11.2	11.5
10	$—NH(CH_2)_2N(C_2H_5)_2$	126–129	A	44	$C_{17}H_{27}N_3O_3$	63.5	63.8	8.5	8.4	13.1	12.8
11	$—NH(CH_2)_2OH$	149–153	A	13	$C_{13}H_{18}N_2O_4$	58.6	58.9	6.8	6.8	10.5	10.9
	(R = C_2H_5—)										
12	—OH	137–138	D	10	$C_{12}H_{15}NO_4$	60.8	61.1	6.4	6.4	5.9	6.3
13	$—OC_2H_5$	[i]		57	$C_{14}H_{19}NO_4$	63.4	63.1	7.2	7.3	5.3	5.7
14	$—NH_2$	208–211	A	67	$C_{12}H_{16}N_2O_3$	61.0	60.3	6.8	6.8	11.9	12.3
15	$—NHC_2H_5$	159–162	A	49	$C_{14}H_{20}N_2O_3$	63.6	64.0	7.6	7.3	...	...
16	$—NH(CH_2)_2N(C_2H_5)_2$	126–128	B	10	$C_{18}H_{29}N_3O_3$	64.5	64.5	8.7	8.8	12.5	12.8

[a] Melting points were established on a Fisher-Johns apparatus. [b] R. S. = recrystallizing solvent; A = acetonitrile; B = water; C = methanol; D = methyl ethyl ketone. [c] Yields are based on recrystallized or distilled product. [d] Analyses were performed by Weiler and Strauss, Oxford, England. [e] Reported, Beilstein XIII, p. 465, m. p. 175–176°. [f] Reported, *ibid.*, m. p. 103–104°. [g] Reported, *ibid.*, m. p. 202–208°. [h] B. p. 186–192° (0.03 mm.). [i] B. p. 182–184° (0.01 mm.).

%) from the cholesterol level at the initiation of the experiment was noted.

Responses showing a hypocholesteremic effect of more than 30% were observed with the following compounds: compound number ($LD_{min.}$): 8 (750), 10 (>1,000), 11 (750), 16 (>1,000), with the highest activity (46% reduction) being noted with compound 16. This compound also afforded a 31% reduction in cholesterol at 60 mg./Kg., p. o.

Structure-activity relationships indicate that for significant hypocholesteremia in I, that R be substituted as lower alkyl, and Z be substituted to provide an amide function. See Table I.

Other pharmacological responses of interest were local anesthetic effect (ED_{50}, 15 mg./ml.) (6) with compound 10, and adrenergic block (6) with compound 13.

EXPERIMENTAL[1]

Ethyl α-(4-Acetamidophenoxy)butyrate (Compound 13).—A mixture of 30.2 Gm. (0.2 mole) of *p*-hydroxyacetanilide, 39.0 Gm. (0.2 mole) of ethyl α-bromobutyrate, and 27.6 Gm. (0.2 mole) of potassium carbonate in 150 ml. of acetone was heated under reflux, with stirring, for ten hours. When cool, the solvent was removed and the residue was dissolved in ether and washed successively with 5% aqueous sodium carbonate and water. After drying (anhydrous magnesium sulfate) and removal of ether, distillation gave 30 Gm. (57%) of product, b. p. 182–184° (0.1 mm.).

[1] Typical procedures are given, data appearing in Table I are not reproduced.

N-(2-Diethylaminoethyl)-α-(*p*-acetamidophenoxy)propionamide (Compound 10).—Ethyl α-(*p*-acetamidophenoxy)propionate (7.0 Gm., 0.02 mole), 16.2 Gm. (0.14 mole) of 2-diethylaminoethylamine, and 0.5 ml. of 25% sodium methylate in methanol were placed in a flask fitted with a 10-cm. fractionating column and distilling head, and heated at 140° for nine hours. The temperature was then raised to 160° and the formed ethanol distilled out. After repeating this heating and distillation procedure, excess amine was removed and the residue on trituration with ether granulated, and on recrystallization (acetonitrile) yielded 4.0 Gm. (44%) of product.

α-(*p*-Acetamidophenoxy)propionic acid (Compound 6).—Ethyl α-(4-acetamidophenoxy)propionate (7.0 Gm., 0.028 mole) was refluxed for four hours with 23.9 ml. (0.028 mole) of 1.17 *N* aqueous sodium hydroxide. When cool, on acidification, the product separated.

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Oleanolic Acid in *Lardizabala biternata*

By MARIO SILVA and PEDRO MANCINELLI

Oleanolic acid was isolated and identified as a sapogenin of a glycosidic fraction from *Lardizabala biternata*. By means of an alcoholic extraction of the dried and pulverized plant an extract was obtained which yielded a water-soluble glycosidic fraction. Acid hydrolysis of this material gave oleanolic acid which was purified and identified.

In connection with a study on saponins of certain typical Chilean species (1, 2), now under way in this laboratory, it appeared of interest to study the *Lardizabalaceae* family. In this same family Mitra and Karrer (3) studied genus *Holboellia* and Kuwada and Fuwa (4) studied genus *Akebia*.

This is a study of the alcoholic extract of the dried and pulverized plant. This extract yielded a water-soluble glycosidic fraction.

Acid hydrolysis of this material yielded an acidic sapogenin which was identified as oleanolic acid and was further characterized through the methyl ester, the acetate, and the benzoate.

EXPERIMENTAL[1, 2]

Isolation of Oleanolic Acid.—Leaves of *Lardizabala biternata* R. et P. collected in May 1959 near Concepción, on the road to Coronel, Chile, were dried for two days at 80–90°. Four kilograms of the dry leaves was ground and extracted continuously in a Sohxlet extractor with benzene to exhaustion. The defatted plant material was dried at 80° and the ethanol-soluble constituents were extracted. This dark green solution was concentrated to a volume of 2 L. and acidified with hydrochloric acid (1240 ml.) to render it approximately 2.5 *N*. The mixture was then refluxed for six hours and poured into water and ice. After twenty-four hours the precipitate was collected on a filter and washed with water, yielding 384 Gm. of a light green mass. The crude oleanolic acid was partially purified by continuous extraction with a 1:1 mixture of benzene-petroleum benzin (b. p. 65–75°), yielding 81 Gm. of white product.

Part of this white product (7.5 Gm.) was chromatographed on 200 Gm. of alumina. Elutions with benzene and chloroform yielded 2.7 Gm. of a yellow oil that was not further investigated. Subsequent applications of chloroform-ethanol (2:1) eluted the remaining material from the column. It weighed 4.73 Gm., and melted between 301–307°. Part of this product (0.674 Gm.) was crystallized from ethanol to yield oleanolic acid with m. p. 305–308°. The ultraviolet spectrum showed evidence of an isolated double bond. The infrared spectrum was identical to that of an authentic specimen.

Anal.—Calcd. for $C_{30}H_{48}O_3$: C, 78.89; H, 10.59. Found: C, 78.65; H, 11.00.

The melting point of the acid was undepressed upon admixture with an authentic specimen.

Oleanolic Acid Acetate.—It was obtained as needles after crystallization from methanol-chloroform; m. p. 263–265°. The infrared spectrum was identical to that of an authentic specimen.

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 77.11; H, 10.40. Found: C, 77.00; H, 9.97.

Oleanolic Acid Methyl Ester.—This ester was prepared by diazomethane treatment in ether solution; crystallized from methanol-chloroform as colorless crystals with m. p. 196–198°.

Anal.—Calcd. for $C_{31}H_{50}O_3$: C, 79.09; H, 10.70. Found: C, 79.50; H, 10.63.

Oleanolic Acid Benzoate.—The pure product was obtained as needles after crystallization from methanol-chloroform; m. p. 259–260°.

Anal.—Calcd. for $C_{37}H_{52}O_4$: C, 79.25; H, 9.34. Found: C, 79.51; H, 9.29.

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The authors wish to acknowledge the stimulation received from The British Council and the help of Dr. K. H. Overton for his suppling of an authentic specimen of oleanolic acid. They are grateful to Mr. R. P. M. Bond for his ultraviolet and infrared analysis done at the Chemistry Department of the University of Glasgow.

[1] Melting points are uncorrected.

[2] We are indebted to the Department of Organic Chemistry of the University of Concepción for the microanalysis of the samples.

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Tests of Amino Acids with Alkaloidal Reagents

By CHARLES L. WINEK and THOMAS J. FITZGERALD

THERE ARE MANY references made in the literature to "false" positive tests obtained with alkaloidal precipitating reagents (1–5). These false tests are attributed to several natural constituents, including amino acids. Since at least one reference (2) lists amino acids as giving a positive test with alkaloidal precipitating reagents while other reference books do not mention amino acids as such, it was felt that the matter should be clarified because of the universal presence of amino acids.

In order to determine whether amino acids do or do not give positive tests with common alkaloidal precipitating reagents this investigation was undertaken. Twenty-seven amino acids were tested with four different alkaloidal precipitating reagents in a qualitative manner. A sample of approximately 100 mg. of each amino acid was dissolved in 2 ml. of 5% hydrochloric acid solution. A volume of 0.5 ml. of this solution was placed in each of four test tubes and the alkaloidal reagent added. The 27 amino acids listed in Table I were tested with 1% platinic chloride solution, gold chloride T.S., Valser's reagent U.S.P., and Dragendorff's reagent.

No precipitate resulted with any of the tests performed, indicating that these amino acids are not capable of giving a positive test with these alkaloidal reagents. Color changes were noticed with two of the amino acids. 3-Aminotyrosine hydrochloride gave an immediate purple color with gold chloride T.S. This purple color gradually turned to a ruby-red. D-Methionine decolorized gold chloride T.S.

One can conclude that amino acids are not as likely to be responsible for false alkaloid tests as the literature leads one to believe.

TABLE I.—AMINO ACIDS TESTED WITH ALKALOIDAL PRECIPITATING REAGENTS

DL-Alanine	DL-Homoserine	D-Tryptophane
L-Asparagine	DL-Isoleucine	DL-Phenylalanine
D-Aspartic acid	DL-Homocystine	DL-Proline
L-Arginine	DL-Leucine	DL-Threonine
L-Cysteine	DL-Lysine	DL-Serine
3-Aminotyrosine HCl	DL-Norleucine	DL-Valine
Cystine	DL-Norvaline	β-Alanine
Histidine	D-Methionine	Glycine
Hydroxy-L-proline	DL-Ornithine HCl	DL-Tyrosine

Received September 5, 1961, from the Ohio State University, College of Pharmacy, Columbus 10.

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The authors wish to thank Dr. Jack L. Beal, College of Pharmacy, for his suggestions, and Dr. William Boyd, Department of Microbiology, for supplying the amino acids used in this study.

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Synthesis and Pharmacology of Several Substituted Indole-3-carboxamide Derivatives

By H. C. WORMSER† and S. ELKIN

Several amides have been prepared which showed oxytocic and local anesthetic activity. These compounds were obtained by reacting indole-3-carbonyl chloride with several amines in ethyl acetate. Indole-3-carbonyl chloride was prepared by the decarbonylation of indole-3-glyoxylyl chloride in tetrachloroethane at 115–120°.

AMIDE DERIVATIVES have found extensive use in medicine (1). Bovet (2) reported that relatively simple substituted aminoacetamides exhibited oxytocic activity in both animals and humans. Also several potent amide-type local anesthetics are known. Among these are included dibucaine, lidocaine, and procainamide. The pharmacological potential of an amide-type local anesthetic is important in that the enzyme pseudocholinesterase destroys the ester-type compound, but has no effect on the amide linkage (1).

Since the indole nucleus appears in many naturally occurring compounds, *viz.* ergot alkaloids, serotonin, reserpine, etc., the pharmacological value resulting from the addition of an amide group to the indole moiety is significant.

Received July 24, 1961, from the Department of Chemistry, Temple University, School of Pharmacy, Philadelphia, Pa.

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Abstracted from a thesis presented by H. C. Wormser to the Graduate School of Temple University in partial fulfillment of the requirements for the degree of Master of Science, 1961.

† Fellow, American Foundation for Pharmaceutical Education.

EXPERIMENTAL

Indole-3-carbonyl Chloride.—Prepared essentially according to the method of Peterson, *et al.* (3), the indole, 20.0 Gm. (0.16 mole), was dissolved in 200 ml. of dry ether and 20 ml. of oxalyl chloride was added slowly to form the corresponding indole-3-glyoxylyl chloride as a bright yellow product. The

latter compound was dissolved in 300 ml. of tetrachloroethane and heated at 115–120° until the rapid evolution of carbon monoxide ceased. The reaction mixture was then allowed to cool rapidly to room temperature and 800 ml. of petroleum ether was added to precipitate the acid chloride. The resulting brown precipitate was filtered, washed with petroleum ether, and dried in a current of dry air. This crude product showed two peaks for the carbonyl grouping in the infrared region (1,750 cm.$^{-1}$ and 1,690 cm.$^{-1}$). It was concluded (3) that two compounds were present in the crude powder obtained: indole-3-carbonyl chloride and a condensation product of the latter, probably a dimer. The product was dissolved in boiling benzene, the solution was filtered, and 50 ml. of petroleum ether was added to the filtrate. The dark brown precipitate which formed was removed by filtration and discarded. Petroleum ether, 150 ml., was added to the now yellow solution and the resulting yellow precipitate collected and dried; m. p. 130° (decompn.). No melting point is given in the literature (3). Yield 5.4 Gm. (24%). This product was identified by hydrolyzing to indole-3-carboxylic acid.

N-(2-Diethylaminoethyl)-indole-3-carboxamide Hydrochloride.—Indole-3-carbonyl chloride, 1.0 Gm. (0.006 mole), was dissolved in 30 ml. of anhydrous ethyl acetate. N,N-Diethylethylenediamine, 1.39 Gm. (0.012 mole), dissolved in 5 ml. of ethyl acetate, was added to the above solution. The reaction mixture was shaken for ten minutes in a separator and then washed several times with water to remove the diamine hydrochloride salt. The ethyl acetate solution was dried over sodium sulfate and the solvent removed under reduced pressure. A light brown oil resulted. After washing this product several times with cold petroleum ether a light tan solid crystallized out slowly. This product was recrystallized from methanol-water to give a white compound having a melting point of 108–109°. The infrared spectrum indicated both N-H and amide structures. A positive Hopkins-Cole test for indole was obtained (propylene glycol). Recrystallization yielded 1.2 Gm. (83.4%). The hydrochloride salt was formed by dissolving the free base in ether and slowly adding, with stirring, a solution of dry hydrochloric acid in cold anhydrous ether. The crude hydrochloride which formed was found to be hygroscopic and had to be recrystallized several times from alcohol-ether to give a pure stable compound, m. p. 146–147°; yield, 0.80 Gm. (48.7%).

Anal.—Calcd. for $C_{15}H_{22}ClN_3O$: N, 14.21; C, 60.92; H, 7.50. Found: N, 14.36; C, 61.61; H, 7.94.

N-(3-Dimethylaminopropyl)-indole-3-carboxamide Hydrochloride.—Prepared in a similar manner to the foregoing, the free base, obtained by reacting 3.0 Gm. (0.018 mole) of indole-3-carbonyl chloride and 3.67 Gm. (0.036 mole) of N,N-dimethylpropylenediamine, melted at 119–122°; yield, 1.42 Gm. (34.6%). The hydrochloride, upon recrystallization from an ethanol-ether mixture, melted at 233–235°; yield, 0.85 Gm. (18.1%).

Anal.—Calcd. for $C_{14}H_{19}ClN_2O$: N, 14.91; C, 59.69; H, 7.15. Found: N, 15.39; C, 60.35; H, 7.23.

1-(3-Indolyl)-4-methylpiperazine Hydrochloride.—Prepared in an analogous manner from 3.0 Gm. (0.018 mole) of indole-3-carbonyl chloride and 2.32 (0.036 mole) of N-methylpiperazine, the free base was recrystallized from aqueous ethanol; m. p. 194°. The hydrochloride melted at 268–270°; yield, 0.96 Gm. (20.6%).

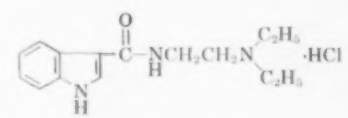
Anal.— Calcd. for $C_{14}H_{19}ClN_2O$: N, 15.02; C, 60.11; H, 6.48. Found: N, 14.55; C, 59.54; H, 6.35.

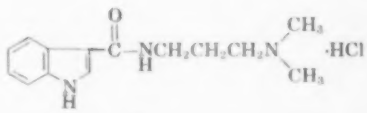
Testing Procedures.—*Local Anesthetic Activity.*—The winking reflex of the rabbit's eye was tested by touching the cornea gently with a von Frey hair. Several drops of the compound were instilled into the conjunctival sac and the time for the winking reflex to return to normal was noted.

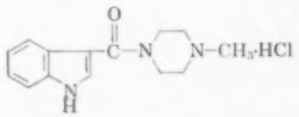
Oxytocic Activity.—Five female rats were injected in the inguinal muscle 24 hours prior to the experiment with 5.5 mcg. of estradiol propionate in peanut oil. The uterus was excised and a strip (approximately two-thirds of the total horn in length) was suspended in a 100-ml. beaker and filled with Tyrode's solution at 30°. This temperature was maintained throughout the experiment. The results were recorded on a revolving smoked drum.

RESULTS

The following substituted amide derivatives of indole have been prepared







All three compounds showed local anesthetic activity. In all cases, the optimum concentration was 2%, lower concentrations had a shorter duration of action, and higher concentrations caused hyperemia. The most effective local anesthetic was N-(2-diethylaminoethyl)-indole-3-carboxamide hydrochloride.

The three compounds, in concentrations of 1 to 2 mg. per 100 ml. of solution, had a pronounced stimulating action on the estrous uterus causing the isolated segment to contract and increase in tone and motility. Epinephrine was added to determine whether the action of the compounds tested was specific and, in each case, a relaxation of the uterine muscle occurred. N-(3-Dimethylaminopropyl)-indole-3-carboxamide hydrochloride showed the most potent oxytocic activity.

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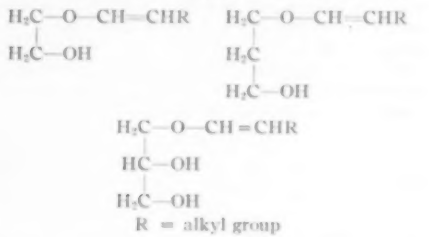
Synthesis of α,β-Unsaturated Ethers of Polyhydric Alcohols and Neutral Plasmalogens

Sir:

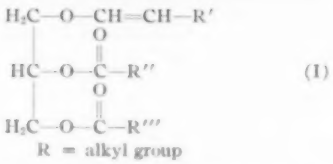
Recent developments in the field of the plasmalogens have produced evidence that the aldehydogenic linkage in the plasmalogens is an α,β-unsaturated ether (1). We have prepared a series of α,β-unsaturated ethers of polyhydric alcohols with the following general formulas



R = alkyl group

The procedure involves a transacetalation reaction (2) between the polyhydric alcohol and an α-bromo dimethylacetal of a straight chain aldehyde. This α-bromo cyclic acetal is then refluxed with sodium in ether to yield the desired compounds. These compounds show an infrared absorption band of relatively strong intensity near 6 μ which is indicative of a —O—CH=CH— grouping (3). The elemental analysis and the 2,4-dinitrophenylhydrazone derivatives were correct. These derivatives formed immediately upon adding the reagent. The compounds show an α,β-unsaturated ether content of 80 to 96% upon assaying with iodine in methanol by the method of Siggia (4). The corresponding saturated ethers were prepared by hydrogenating the α,β-unsaturated ethers. As a means of structure proof one of the saturated ethers, 3-octyloxy-1,2-propanediol was prepared in a different manner. This consisted of reacting the sodium salt of isopropylidene glycerol with octyl bromide and then cleaving the ketal structure by acid hydrolysis. The physical constants of the two saturated ethers agreed favorably. The saturated ethers of glycerol gave a positive periodic acid test substantiating the α-position of the α,β-unsaturated ether linkage on the glycerol moiety.

In addition, several neutral plasmalogens (5) have been synthesized by a reaction of the above α,β-unsaturated ethers of glycerol with long chain acid chlorides in the presence of pyridine. A general plasmalogen formula:



(I)

R = alkyl group

The constants for 3-(1-octenyloxy)-1,2-propanediol are presented as typical for the entire series of α,β-unsaturated ethers prepared. A product, b_1 135–138°, n_D^{27} 1.4670 was obtained.

Anal.—Calcd. for $C_{11}H_{22}O_3$: C, 65.30; H, 10.96. Found: C, 65.19; H, 11.15. Infrared spectrum: 2.9 μ (O—H), 3.4 μ (C—H), 6 μ (—O—HC=CH—), 6.84 (C—H). The melting point of the 2,4-dinitrophenylhydrazone was found to be 95–96°. This agrees favorably with the literature (6).

The constants for 3-octyloxy-1,2-propanediol synthesized by hydrogenating the above compound are as follows: $b_{0.65}$ 135–136°, n_D^{25} 1.4503.

Anal.—Calcd. for $C_{11}H_{24}O_3$: C, 64.66; H, 11.84. Found: C, 64.29; H, 11.88. Infrared spectrum: 2.9 μ (O—H), 3.4 μ (C—H), 6.84 (C—H).

The constants for 3-octyloxy-1,2-propanediol synthesized by the alternate method are as follows: $b_{0.65}$ 130°, n_D^{25} 1.4490.

Anal.—Calcd. for $C_{11}H_{24}O_3$: C, 64.66; H, 11.84. Found: C, 64.70; H, 11.88. Infrared spectrum: 2.9 μ (O—H), 3.4 μ (C—H), 6.84 (C—H).

Compound (I), in which R′ is a hexyl radical, and R″ and R‴ are pentadecyl radicals, was found to be a low melting, white, waxy solid. Further work on these compounds is in progress in this laboratory.

Anal.—Calcd. for $C_{43}H_{82}O_5$: C, 76.05; H, 12.17. Found: C, 75.89; H, 12.24.

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Book Notices

Index Handbook of Cardiovascular Agents. Vol. 2, Parts 1 and 2. Edited by ISAAC D. WELT. National Academy of Sciences—National Research Council, Washington, D. C., 1960. lv + 1568 pp. 22.5 × 27.5 cm.

The bibliographic portion of this comprehensive documentation effort lists about 13,400 scientific communications concerned with drugs acting upon the heart and circulation. The author index occupies 112 pages, and the remaining 1,150 pages (includes Part 2) contain the indexed and cross-indexed subject material. It is an outstanding contribution to the scientific reference library.

Drug Standardization Information Service. Edited by K. GERECKE, L. KNY, and J. RICHTER. Staatliches Institut fur Arzneimittelprufung, Berlin-Weissensee, den Grosse Seestrasse 4, 1960. 78 pp. + card-style references. 16 × 23 cm. Paperbound.

Articles pertaining to drug standardization submitted in German, Russian, or English are printed in the original language with summaries in the three languages. Specifications for individual compounds are given and brief descriptions of other compounds are given on pages marked for cut out as cards for filing.

Recent Data on Pharmacology and Clinic of the Phenothiazine Derivatives. Transactions. Vol. 1. Edited by V. V. SAKUSOV. Academy of Medical Sciences of the U. S. S. R., Moscow, 1958. 188 pp. 16.5 × 24 cm. (Russian.)

The reports in this book are in the Russian language with a brief English summary of each paper.

Quantitative Methods in Pharmacology. Edited by H. DE JONGE. Interscience Publishers, Inc., 250 Fifth Ave., New York, N. Y., 1961. xx + 391 pp. 15 × 22 cm. Price $13.25.

The proceedings of a symposium sponsored by the Biometric Society and held at Leyden, Holland, May 10–13, 1960, are divided under the headings: Sequential analysis, Standardization of drugs, Parametric or nonparametric statistical methods, Screening of drugs, Mixtures of drugs, Miscellaneous topics. The compilation is not indexed.

The Actinomycetes. Vol. 2, Classification, Identification and Descriptions of Genera and Species. By SELMAN A. WAKSMAN. The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1961. ix + 363 pp. 17 × 25.5 cm. Price $15.

An authoritative compilation dealing with the characteristics of the well recognized genera of the actinomycetes. Various closely related genera that are frequently included in the order *Actinomycetales*, notably the genus *Mycobacterium* Lehmann and Neumann, 1896, are not considered. An index of organisms and a general index are appended.

Chemistry of Drug Metabolism. By WILLIAM H. FISHMAN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1961. xvii + 235 pp. 15 × 23 cm. Price $10.50.

The metabolic transformations of organic chemicals and drugs, and the biochemical mechanisms indicated in the metabolism of certain drugs are coordinated in this monograph in the series "American Lectures in Living Chemistry," edited by I. Newton Kugelmass.

Advances in Pest Control Research. Vol. 4. Edited by R. L. METCALF. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1961. vi + 3477 pp. 15 × 22.5 cm. Price $12.50.

Subjects included in this volume are: Some fundamental aspects of applied insect pathology, Synthetic pyrethroids, Selective toxicity of insecticides, Potentiation of the toxicity of organophosphorus compounds, Chemical control of insect pests of domestic animals, Residue determination by cholinesterase inhibition analysis, Principles of aquatic plant control, and A digest of available information on the insecticide malathion. References are given at the end of each chapter, with 658 references for the chapter on malathion. A subject index for volume 4, and a cumulative table of contents for volumes 1–4 are appended.

Annual Review of Pharmacology. Vol. 1. Edited by WINDSOR C. CUTTING, ROBERT H. DREISBACH, and HENRY W. ELLIOTT. Annual Reviews, Inc., Palo Alto, Calif., 1961. vii + 479 pp. 15 × 22 cm. Price $7 U. S., $7.50 elsewhere.

Volume 1 of this addition to the family of Annual Reviews includes chapters on the following subjects: Highlights of pharmacology in Japan, in Latin America, in the U. S. S. R., Mechanisms of drug absorption and distribution, Metabolic fate of drugs, Effects of temperature on drug action, Biochemical effects of drugs, Recent observations on hypersensitivity to drugs and use of drugs in allergy, Methods for studying the behavioral effects of drugs, Behavioral pharmacology, Pharmacologically active substances of mammalian origin, Pharmacology of autonomic ganglia, Neuromuscular pharmacology, Cardiovascular pharmacology, Renal pharmacology, Endocrine pharmacology: selected topics, The action of drugs on the skin, The pharmacology and toxicology of the bone seekers, and Toxicology of Organic compounds of industrial importance. A prefatory chapter by Torald Sollmann includes a brief autobiographical sketch. Author and subject indexes are appended.

The Electron Microscope. By M. E. HAINE and V. E. COSSLETT. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1961. xvi + 282 pp. 14 × 21.5 cm. Price $9.25.

The Present State of the Art is the subtitle of this book which deals with the theory and practice of the electron microscope. It is intended as a working text on the subject.

Calculations in Pharmacy. By SUE H. ROUSE and M. GEORGE WEBBER. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1961. xi + 234 pp. 15 × 23 cm. Price $5.

The authors observe that "The subject matter usually presented in the study of pharmaceutical calculations is not sufficiently complex to account for the number of students who have been failing such a course in the pharmaceutical curriculum." We hope this new presentation of the subject will give the correct answer to this perplexing problem.

Metabolic Effects of Adrenal Hormones. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1960. viii + 109 pp. 12.5 × 18.5 cm.

Five papers on subjects related to adrenal steroids and the discussions of the reports at the 1960 meeting of the Ciba Foundation Study Group No. 6 are presented in this book.

Essential Hypertension. Edited by K. D. BOCK and P. T. COTTIER. Springer-Verlag, Reichpietschufer 20, Berlin W 35, German, 1960. viii + 392 pp. 13.5 × 20.5 cm.

Twenty-two reports on various aspects of essential hypertension, covering theoretical views, the nature and the therapy of this condition, are included in this international symposium. Group discussions are included. A subject index is appended.

The Encyclopedia of the Biological Sciences. Edited by PETER GRAY. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1961. xxi + 1119 pp. 17 × 25 cm. Price $20.

More than 800 contributed articles of 500 to 5,000 words cover the broad field of the biological sciences in this book. The clearly explained method of indicating cross references in the text and in the general index add greatly to its reference value.

Pharmacodynamie Biochimique. 12th ed. By Z. M. BACQ, J. CHEYMOL, M. J. DALLEMAGNE, R. HAZARD, J. LA BARRE, J. J. REUSE, and M. WELSCH. Masson et Cie., 120, boulevard Saint-Germain, Paris 6e, 1961. xvi + 1227 pp. 16 × 24 cm. Price 140 NF.

This textbook (in French) is divided into two main sections: I. General pharmacodynamics (112 pages), and II. Specific pharmacodynamics. The second part is subdivided under the headings: Biogenous amines and the autonomic nervous system, Hormones, Vitamins, Proteins, lipids and essential factors, Carriers of sulfhydryls and antagonists, Anions and cations, chemotherapy, Principal systems and medicinal regulation of their functions, Poisons used against insects, rodents, and mollusks. A general index is appended.

The Friel System. A Language of Color. By EDWARD FRIEL. Edward Friel, 1837-12th Ave. West, Seattle, Wash., 1961. iv + 79 pp. 22 × 27.5 cm. Paperbound.

This book represents a unique and original approach to the development of a color system.

Actualités Pharmacologiques. 13th series. Edited by RENÉ HAZARD and JEAN CHEYMOL. Masson et Cie. 120, boulevard Saint-Germain, Paris 6e, 1960. 266 pp. 16.5 × 25 cm. Paperbound. Price 42 NF.

The subjects covered by contributors in this volume (in French) are: Pharmacological actions on the biliary tract, Electroencephalographic studies on pharmacological reactions of the paleencephalon, Status of curarizing substances and modifiers of curarization, Histamine-liberating substances and processes of endogenous histamine liberation, Antagonists of peripheral actions of 5-hydroxytryptamine, Pharmacodynamics of thiamine and derivatives, Pharmacothesaurismosis, and Toxicity of edema from burns.

Antibiotica. By TADENSZ KORZYBSKI and WLODZIMIERZ KURYLOWICZ. Veb Gustav Fischer Verlag, Jena, Germany, 1961. xix +1105 pp. 16.5 × 24 cm. Price DM 130.

Antibiotics are arranged in this book (in German) according to a classification of the microorganisms with which their origin is associated. Descriptions, properties, and applications (if any) are reported; with literature references after each entry. A general author index, an index of antibiotics, and an index of microorganisms which yield antibiotics are appended.

Medical Pharmacology. By ANDRES GOTH. The C. V. Mosby Co., 3207 Washington Blvd., St. Louis 3, Mo., 1961. 551 pp. 17 × 24.5 cm. Price $11.

Principles and concepts rather than factual data form the basis for this textbook on pharmacology for medical students and physicians. Essential facts about important drugs are included, while only essential differences between its congeners and the prototype are discussed. An index is appended.

The Chemistry and Mode of Action of Herbicides. By A. S. CRAFTS. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1961. viii + 269 pp. 15 × 22.5 cm. Price $9.

Intended as a reference textbook for herbicide researchers, this book starts with chemical and application classifications of compounds used as herbicides. Selective and nonselective herbicides and the physiology of herbicidal action are discussed, then the more important useful compounds are considered. A weed index and a subject index are appended.

Cancer Chemotherapy. Prepared under the direction of R. LEE CLARK, Jr. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1961. xii + 253 pp. 15 × 23 cm. Price $10.50.

A useful review of known anticancer drugs and hormones, the book includes 382 references to current chemotherapeutic research publications indexed by general references, bibliographic sources, disease categories, and drugs. For clinicians, the text covers comparative effectiveness of the agents: When to apply a drug, average dose, when to discontinue a drug, past results, and chemical background. A general index also is appended.

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